CERTIFICATE OF ELECTRONIC TRANSMISSION
37 C.F.R. § 1.8

I hereby certify that this correspondence is being electronically filed with the United States Patent and Trademark Office via EFS-Web on the date below:

May 7, 2007
Date

David L. Parker

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Gong et al.	Group Art Unit: 1632
Serial No.: 10/605,708	Examiner: Singh, Anoop Kumar
Filed: October 21, 2003	Atty. Dkt. No.: GLOF:007USC1
For: CHIMERIC GENE CONSTRUCTS FOR GENERATION OF FLUORESCENT TRANSGENIC ORNAMENTAL FISH	

APPEAL BRIEF

MS Appeal Briefs
Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Appellants hereby submit this Appeal Brief to the Board of Patent Appeals and Interferences pursuant to 37 C.F.R. §41.31(a)(1) and 41.37 in light of the Final Office Action dated November 3, 2006 and Notice of Appeal dated February 5, 2007.

A request for a one month extension of time to respond is included herewith along with the required fee. This one month extension will bring the due date to May 5, 2007, which is within the six-month statutory period. Should such request or fee be deficient or absent, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/GLOF:007USC1.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, National University of Singapore. The subject matter of the application is currently licensed to Yorktown Technologies, Inc.

II. RELATED APPEALS AND INTERFERENCES

There are no interferences or appeals for related cases.

III. STATUS OF THE CLAIMS

Claims 1-3, 9-28 and 30-42 are pending, of which claims 17-19, 22-23, 25-28, 33 and 34 are withdrawn. Claims 1-3, 9-16, 20-21, 24, 30-32 and 35-42 are subject to the current appeal.

IV. STATUS OF AMENDMENTS

An amendment dated February 5, 2007, was filed in order the place the claims in better form and reduce the issues for appeal in order to remove the prior art rejections from consideration. Applicants reserve the right to proceed with the cancelled subject matter in future continuing applications. The Examiner was contacted on May 3, 2007, and he indicated that he had not yet received the amendment and, thus, the amendment has not yet been considered as of May 3, 2007.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

Please note that since the present application was filed electronically using the earlier electronic submissions software, the application as filed has no "page numbers" and "line numbers" per se, hence, Appellants will refer below to the paragraph numbers where the relevant supporting information can be found in the specification.

Independent claim 1 is directed to a method of providing transgenic fish to the ornamental fish market, comprising the steps of obtaining a transgenic fish comprising one or more fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses one or more fluorescent proteins selected from the group of fluorescent proteins consisting of a blue fluorescent protein, a yellow fluorescent protein and a cyan fluorescent protein, encoded by the one or more fluorescence genes; and distributing said fish to the ornamental fish market. Support can be found, *e.g.*, in paragraphs [0009], [0010], [0040], [0091], [0092].

Separately argued dependent claims 20 and 21 are directed to the zebrafish muscle creatine kinase gene promoter and zebrafish myosin light chain 2 gene promoter. Support can be found in paragraphs [0012], [0033], [0060], [0070] and [0078] (each of the foregoing for creatine kinase promoter), and [0012], [0034], [0060], [0061], [0072] and [0078] (each for the myosin light chain promoter).

Separately argued dependent claims 39 and 40 concern the method of claim 1 or 15, wherein the transgenic fish is a transgenic zebrafish, medaka, goldfish or carp (claim 39) or the method of claim 36, wherein the second fish is a zebrafish, medaka, goldfish or carp (claim 40). Support can be found in paragraph [0096].

Separately argued dependent claim 41 concerns the method of claim 1 or 36,wherein the transgenic fish is a transgenic koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus, hatchet fish, Molly fish, or pangasius. Support can be found in paragraph [0096].

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

There are four rejections that are the subject of the present appeal:

1) A rejection of claims 1-16, 20-21, 29-32 and 35-42 as lacking written description under 35 U.S.C. §112, first paragraph; and

2) A rejection of claims 1-16, 20-21, 29-32 and 35-42 as lacking enablement under 35 U.S.C. §112, first paragraph.

VII. ARGUMENT

A. Section 112, First Paragraph, Rejection (Written Description)

The Action first enters a written description rejection of all of the claims on the basis that the specification is said to fail to describe "a transgenic fish comprising one or more fluorescence genes positioned under the control of any promoter" taking the position that the specification only describes a transgenic fish comprising one or more fluorescent proteins encoded by one or more fluorescence genes at a level sufficient such that said fish fluoresces upon exposure to one or more of a blue light, ultraviolet light or sunlight. Appellants disagree.

First, the Applicants note an error in the Examiner's reasoning. The Examiner states at the bottom of page 4 that the claims "do not require that the transgenic fish show any fluorescence." While not considered to be relevant to written description, this is incorrect. The claims states that the fish must express the fluorescent protein(s) so, by definition, the fish will fluoresce.

With respect to the Examiner's contention that the specification is limited to the use of a promoter that expresses the genes "at a level sufficient such that said fish fluoresces upon exposure to one or more of a blue light, ultraviolet light or sunlight," there is simply no basis set

forth for the rejection. The Action fails to identify the language from the specification that purportedly limits the written description. On the contrary, while the specification does indicate that the most preferred transgenic zebrafish are those whose fluorescent color is observed when the fish are exposed to sunlight (see, *e.g.*, [0085]), there is no such boundary placed on the invention by the subject specification. Exemplary excerpts in this regard include:

> [0010] It is another objective of the invention to develop fluorescent transgenic ornamental fish using these gene constructs. By applying different gene promoters, tissue-specific, inducible under different environmental conditions, or ubiquitous, to drive the GFP gene, GFP could be expressed in different tissues or ubiquitously. Thus, these transgenic fish may be skin fluorescent, muscle fluorescent, ubiquitously fluorescent, or inducibly fluorescent. These transgenic fish may be used for ornamental purposes, for monitoring environmental pollution, and for basic studies such as recapitulation of gene expression programs or monitoring cell lineage and cell migration. These transgenic fish may be used for cell transplantation and nuclear transplantation or fish cloning.
> ...
>
> [0040] Specifically Exemplified Polypeptides/DNA. The present invention contemplates use of DNA that codes for various polypeptides and other types of DNA to prepare the gene constructs of the present invention. DNA that codes for structural proteins, such as fluorescent peptides including GFP, EGFP, BFP, EBFP, YFP, EYFP, CFP, ECFP and enzymes (such as luciferase, .beta.-galactosidase, chloramphenicol acetyltransferase, etc.), and hormones (such as growth hormone etc.), are useful in the present invention. More particularly, the DNA may code for polypeptides comprising the sequences exemplified in SEQ ID NOS:2, 4, 6 and 21. The present invention also contemplates use of particular DNA sequences, including regulatory sequences, such as promoter sequences shown in SEQ ID NOS: 7, 8, 9 and 22 or portions thereof effective as promoters. Finally, the present invention also contemplates the use of additional DNA sequences, described generally herein or described in the references cited herein, for various purposes.

Accordingly, there is no basis on this record for maintaining the rejection.

B. Section 112, First Paragraph, Rejection (Enablement)

The Action next maintains an enablement rejection against all of the claims, taking the position that the specification fails to enable the use of any promoter in the preparation of any fish.

"Preparation of any fish"

With respect to the "preparation of any fish" issue, Appellants first note that many, many different species of fish have now been genetically engineered such that now the genetic engineering of fish is generally routine. This is noted in the present specification at paragraph 5:

> [0005] Fish are also an intensive research subject of transgenic studies. There are many ways of introducing a foreign gene into fish, including: microinjection (e.g. Zhu et al., 1985; Du et al., 1992), electroporation (Powers et al., 1992), sperm-mediated gene transfer (Khoo et al., 1992; Sin et al., 1993), gene bombardment or gene gun (Zelemin et al., 1991), liposome-mediated gene transfer (Szelei et al., 1994), and the direct injection of DNA into muscle tissue (Xu et al., 1999). The first transgenic fish report was published by Zhu et al. (1985) using a chimeric gene construct consisting of a mouse metallothionein gene promoter and a human growth hormone gene. Most of the early transgenic fish studies have concentrated on growth hormone gene transfer with an aim of generating fast growing "superfish". A majority of early attempts used heterologous growth hormone genes and promoters and failed to produce gigantic superfish (e.g. Chourrout et al., 1986; Penman et al., 1990; Brem et al., 1988; Gross et al., 1992). But enhanced growth of transgenic fish has been demonstrated in several fish species including Atlantic salmon, several species of Pacific salmons, and loach (e.g. Du et al., 1992; Delvin et al., 1994, 1995; Tsai et al., 1995).

Furthermore, this fact has been recognized by the US PTO in several issued US patents that are presumptively fully enabling. For example, Applicants make reference to Cooper *et al.*, US 5,998,698 [Exhibit 1]. This patent is directed to transgenic fish that are capable of expressing heterologous lytic peptides. This patent provides a detailed disclosure of the preparation of numerous species of transgenic fish, including any bony fish, exemplified by catfish and koi. Note that the claims of this patent cover any transgenic bony fish. Similarly, Winn *et al.*, US 6,307,121 [Exhibit 2], discloses and claims the preparation of any transgenic fish species, including cartilagenous fish, for mutation detection. Please see the first several paragraphs of the Detailed Description section, as well as the claims. Furthermore, we would direct the Board's attention to Winn, US 6,472,583 [Exhibit 3], which similarly discloses and

claims the preparation of any species of transgenic fish, again for mutation detection. These patents are presumptively enabling for the preparation of any transgenic fish.

The evidence put forward by the Examiner does not support the position of non-enablement. The relevance of the Betancourt *et al.* reference [Exhibit 4] is not at all understood, as this reference, which is of limited relevance anyway due to the fact it is very old, actually supports enablement. Betancourt *et al.* merely stands for the proposition that non-fish regulatory elements such as promoters do not work *as well* in fish cells as fish regulatory elements. It does not state that such regulatory elements lack utility in fish, they simply do not work *as well*. Indeed, the abstract states that the CMV promoters and RSV promoters, which are both viral, non-fish, promoters, "were the most potent in all cell types." Note that the excerpt relied upon by the Examiner merely states that transgenic fish cells "should preferably contain" DNA sequences from fish genes – not that *only* fish elements will work. This reference actually demonstrates the level of skill in the art only as of 1993, and shows that even then those of skill were well aware that fish promoters and elements were preferred but not the only elements that would function.

The Bearzotti *et al.* reference [Exhibit 5] is similarly very old and outdated (1992), but again actually supports enablement. It is noted that this reference shows that the CMV, RSV and even the heat-inducible human HSP-70 promoters functioned well in fish cells. Again, the excerpt relied on by the Examiner merely indicates that certain elements may not function as efficiently as others. Moreover, the fact that something "does not work as well", does not lead to the conclusion that the present invention is not enabled for the preparation of any transgenic fish using any promoter. Here Appellants make reference to the "Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, First Paragraph-Enablement of

Chemical/Biotechnical Applications," found on the PTO website at http://www.uspto.gov/web/offices/pac/dapp/1pecba.htm wherein examiner's are reminded that "it is not necessary for an invention to work well to be enabled."

The same can be said for Higashijima *et al.* [Exhibit 6], which again merely stands for the proposition that those of skill in the art were aware that certain design choices may need to be made in order to achieve an optimally performing transgenic fish. This is not a proper basis for raising an enablement concern. Indeed, this reference, which has been relied on by the Examiner in prior art rejections, fully evidences the broad knowledge in the art for making fluorescent transgenic fish have a variety of elements and promoters.

We would also refer the Board to various articles of record. For example, the Examiner is referred to Kuo *et al.*, reference C45 [Exhibit 7], which demonstrates that cis-acting elements from mouse carcinoma nectin neurofilament gene were effective in directing either neuron-specific or skin-specific expression (depending on the particular construct) in zebrafish. See also, Kim *et al.*, reference C44 [Exhibit 8].

The Board is further referred to Moss *et al.*, reference C54 [Exhibit 9], which demonstrates the ability of a rat myosin promoter to direct muscle specific expression in zebrafish.

Lastly, the Board is referred to the review article of Hackett *et al.*, "The Molecular Genetics of Transgenic Fish," [Exhibit 10] which was published in 2000 at about the same time as the filing of the present application. This review article sets forth an abundance of non-fish tissue specific and other promoters that were all routinely found to be operable in fish. See, for example, Tables 1 through 3.

Accordingly, it is submitted that the Examiner has failed to make a *prima facie* case of non-enablement. Further, Applicants have presented substantial evidence in support of broad enablement. Accordingly, the Examiner is requested to withdraw the rejection.

Claims 39-42 (fish species claims)

Claims 39 to 42 should be considered separately as it is even more evident that the fish species set forth in these claims are fully enabled. Claims 39-40 are directed to zebrafish, medaka, goldfish or carp, whereas claim 41 is directed specifically to transgenic koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus, hatchet fish, Molly fish, or pangasius. All of the foregoing are fish that are well studied and, as evidenced by the patents and articles cited above, can be routinely prepared without undue experimentation.

Lastly, claim 42 is directed specifically to transgenic zebrafish. This claim should be acceptable to the Examiner, at least with respect to the "fish species" aspect of the enablement rejection.

"Any promoter"

With respect to the "any promoter" aspect of the rejection, Appellants first note that the Examiner has withdrawn the written description rejection, agreeing that the claims fully satisfy the written description requirement with respect to any promoter, agreeing that the specification adequately describes any promoter generally known in the art. Action at 16. While admittedly a "different" rejection, we believe that this recognition on the written description side of section 112 has relevance to the enablement consideration discussed below.

The Examiner's basis for maintaining the enablement rejection as to the "any promoter" issue is not entirely understood. The Examiner frames the issue at the top of page 15 by stating

that the "issue is whether applicants are enabled for a method of providing a transgenic ornamental fish by obtaining a fish that expresses fluorescent gene at a level sufficient such that said fish fluoresces upon exposure to the light." (emphasis in original). In framing the argument, though, the Examiner misstates the claim wording – the claims merely state that "the transgenic fish expresses one or more fluorescent proteins ... encoded by the one or more fluorescence genes." There is no requirement here that the fish express at any particular level. The Examiner thus reads in a requirement that is not there! We would note that if the transgenic fish do not express the fluorescence at a high level they may well not be very desirable to the ornamental fish market, and may not be well received commercially, but this is not an enablement issue, it is a marketing issue. Again, it is not necessary that an invention work well to be enabled.

The Examiner also places some weight on the fact that Applicant's specification states that with that some heterologous promoters from SV40 and RSV that are used to study zebrafish expression in many cases show variable and unpredictable expression. Again, we fail to see how this observation leads to a conclusion of non-enablement – the fact that the "pattern" (i.e., tissue pattern) of expression using viral promoters is in some cases unpredictable is not of particular relevance. If a particular promoter does not work well for a particular desired application, one of skill in the art – who is obviously very highly skilled as evidenced by the above documents – would simply choose to use a different promoter! The law does not require, as the Examiner appears to argue, that every promoter work well for every desired application. The law simply requires that one of skill be enabled to carry out the invention to determine those embodiments that worked for the desired purpose without undue experimentation. See *In re Angstadt*, 537 F.2d 498, 502-03, 190 USPQ 214, 218 (CCPA 1976); and MPEP Section 2164.08(b). We would also refer to a discussion of the law found in the above-referenced examiner's guidelines:

2. Inoperability/Inoperative Species within the Scope of the Claim

> The presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled. The standard is whether a skilled person could determine which embodiments that were conceived, but not yet made, would be inoperative or operative with expenditure of no more effort than is normally required in the art. Atlas Powder Co. v. E.I. duPont de Nemous & Co., 750 F.2d 1569, 1577, 224 USPQ 409, 414 (Fed. Cir. 1984)(prophetic examples do not make the disclosure non-enabling). Although, typically, inoperative embodiments are excluded by language in a claim the scope of the claim may still not be enabled where undue experimentation is involved in determining those embodiments that are operable. A disclosure of a large number of operable embodiments and the identification of a single inoperative embodiment did not render a claim broader than the enabled scope because undue experimentation was not involved in determining those embodiments that were operable. In re Angstadt, 537 F.2d 498, 502-03, 190 USPQ 214, 218 (CCPA 1976); and MPEP Section 2164.08(b). However, claims reading on significant numbers of inoperative embodiments would render claims nonenabled when the specification does not clearly identify the operative embodiments and undue experimentation is involved in determining those that are operative. Atlas Powder Co. v. E.I. duPont de Nemous & Co., 750 F.2d 1569, 1577, 224 USPQ 409, 414 (Fed. Cir. 1984); In re Cook, 439 F.2d 730, 735, 169 USPQ 298, 302 (CCPA 1971); see also MPEP Section 2164.08(b).

Section III.B.2., Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, First Paragraph-Enablement of Chemical/Biotechnical Applications.

There has been no showing on this record that one of skill would not be able to readily overcome problems of variable and unpredictable patterns of expression where such was desired. On the contrary, the evidence adduced clearly demonstrates that overcoming such problems were routine in the art as of the filing date.

For the foregoing reasons, the Examiner's enablement rejection should be overturned.

Claims 20 and 21

Claims 20 and 21 specify particular zebrafish promoters described and enabled in the specification and thus should certainly be removed form any concern regarding promoter enablement.

VIII. CONCLUSION

Appellants believe that the foregoing remarks fully respond to all outstanding matters for this application. Appellants respectfully request that the Board reverse the rejections of all claims.

Respectfully submitted,

David L. Parker
Reg. No. 32,165
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 474-5201
(512) 536-4598 (facsimile)

Date: May 7, 2007

I. CLAIMS APPENDIX

1. A method of providing transgenic fish to the ornamental fish market, comprising the steps of:

(a) obtaining a transgenic fish comprising one or more fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses one or more fluorescent proteins selected from the group of fluorescent proteins consisting of a blue fluorescent protein, a yellow fluorescent protein and a cyan fluorescent protein, encoded by the one or more fluorescence genes; and

(b) distributing said fish to the ornamental fish market.

2. The method of claim 1, further comprising displaying said transgenic fish under a blue or ultraviolet light.

3. The method of claim 2, wherein the transgenic fish are displayed under an ultraviolet light that emits light at a wavelength selected to be optimal for the fluorescent protein or proteins.

9. The method of claim 1, wherein the transgenic fish express a BFP.

10. The method of claim 9, wherein the transgenic fish express an EBFP.

11. The method of claim 1, wherein the transgenic fish express a YFP.

12. The method of claim 11, wherein the transgenic fish express an EYFP.

13. The method of claim 1, wherein the transgenic fish express a CFP

14. The method of claim 13, wherein the transgenic fish express an ECFP.

15. A method of providing transgenic fish to the ornamental fish market, comprising the steps of:

(a) obtaining a transgenic fish comprising fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses more than one color of fluorescent protein encoded by the fluorescence genes; and

(b) distributing said fish to the ornamental fish market.

16. The method of claim 1 or 15, wherein the promoter is a tissue specific promoter.

20. The method of claim 19, wherein the promoter is a zebrafish muscle creatine kinase gene promoter.

21. The method of claim 19, wherein the promoter is a zebrafish myosin light chain 2 gene promoter.

24. The method of claim 1 or 15, wherein the promoter is a ubiquitously expressing promoter.

25. The method of claim 24, wherein the promoter is a zebrafish acidic ribosomal protein gene promoter.

30. The method of claim 15, wherein the more than one fluorescent protein is expressed in the same tissue, to effect a new fluorescent color.

31. The method of claim 30, where the transgenic fish expresses a GFP and a BFP.

32. The method of claim 15, wherein the more than one fluorescent proteins are separately expressed in different tissues.

35. The method of claim 32, wherein the transgenic fish expresses a YFP under the control of a muscle specific promoter.

36. The method of claim 1 or 15, wherein the transgenic fish is a stable transgenic fish line obtained by a method comprising the steps of:

(a) obtaining a transgenic fish comprising one or more fluorescence genes positioned under the control of a promoter, wherein the transgenic fish expresses one or more fluorescent proteins encoded by the one or more fluorescence genes; and

(b) breeding the transgenic fish with a second fish to obtain offspring; and

(c) selecting from said offspring a stable transgenic line that expresses one or more fluorescent proteins.

37. The method of claim 36, wherein the second fish is a wild type fish.

38. The method of claim 36, wherein the second fish is a second transgenic fish.

39. The method of claim 1 or 15, wherein the transgenic fish is a transgenic zebrafish, medaka, goldfish or carp.

40. The method of claim 36, wherein the second fish is a zebrafish, medaka, goldfish or carp.

41. The method of claim 1 or 36, wherein the transgenic fish is a transgenic koi, loach, tilapia, glassfish, catfish, angel fish, discus, eel, tetra, goby, gourami, guppy, Xiphophorus, hatchet fish, Molly fish, or pangasius.

42. The method of claim 39, wherein the transgenic fish is a transgenic zebrafish.

EVIDENCE APPENDIX

Exhibit 1 –Cooper *et al.*, U.S. 5,998,698, made of record in the Amendment and Response to Office Action dated 08/08/06

Exhibit 2 –Winn *et al.*, U.S. 6,307,121, made of record in the Amendment and Response to Office Action dated 08/08/06

Exhibit 3 –Winn *et al.*, U.S. 6,472,583, made of record in the Amendment and Response to Office Action dated 08/08/06

Exhibit 4 –Betancourt *et al.*, made of record in the Office Action dated 03/08/06

Exhibit 5 –Bearzotti *et al.*, made of record in the Office Action dated 03/08/06

Exhibit 6 – Higashijima *et al.*, made of record in the Office Action dated 03/08/06

Exhibit 7 –Kuo *et al.*, made of record in the Amendment and Response to Office Action dated 08/08/06

Exhibit 8 –Kim *et al.*, made of record in the Amendment and Response to Office Action dated 08/08/06

Exhibit 9 –Moss *et al*, made of record in the Amendment and Response to Office Action dated 08/08/06

Exhibit 10 –Hackett *et al.*, made of record in the Amendment and Response to Office Action dated 08/08/06

EXHIBIT 1

US005998698A

United States Patent [19]

Cooper et al.

[11] Patent Number: 5,998,698

[45] Date of Patent: Dec. 7, 1999

A5

[54] **TRANSGENIC FISH CAPABLE OF EXPRESSING EXOGENOUS LYTIC PEPTIDES**

[75] Inventors: **Richard K. Cooper; Frederick M. Enright**, both of Baton Rouge, La.

[73] Assignee: **Board of Supervisors of Louisiana State University and Agricultural and Mechanical College**, Baton Rouge, La.

[21] Appl. No.: **08/491,609**

[22] Filed: **Jun. 7, 1995**

Related U.S. Application Data

[63] Continuation-in-part of application No. PCT/US94/07456, Jun. 30, 1994, which is a continuation-in-part of application No. 08/085,746, Jun. 30, 1993, abandoned, and a continuation-in-part of application No. 08/084,879, Jun. 30, 1993, abandoned.

[51] **Int. Cl.**[6] **C12N 15/00**

[52] **U.S. Cl.** **800/20**

[58] **Field of Search** ..... 435/6, 69.1, 172.3, 435/325; 935/6, 9, 34, 55, 70; 800/2, DIG. 1, DIG. 4, 20; 536/23.7, 24.1

[56] **References Cited**

FOREIGN PATENT DOCUMENTS

8900199	1/1989	WIPO	C12N 15/00
9012866	11/1990	WIPO	C12N 1/06
9501095	1/1995	WIPO	A01K 67/00
9501424	1/1995	WIPO	C12N 5/10

OTHER PUBLICATIONS

U.S. Ser. No. 373,623, Jaynes et al., filed Jun. 29, 1989.
U.S. Ser. No. 646,449, Jaynes et al., filed Jan. 25, 1991.
U.S. Ser. No. 085,282, Cooper et al., filed Jun. 30, 1993.
Xanthopoulas et al., "The structure of the gene for cecropin B, an antibacterial immune protein from *Hyalophora cecropia*," Eur. J. Biochem., vol. 172, pp. 371–376 (1988).
Kaling et al., "Liver–Specific Gene Expression: A–Activator–Binding Site, a Promoter Module Present in Vitellogen and Acute–Phase Genes," Mol. Cell. Biol., vol. 11, pp. 93–101 (1991).
Boman et al., "Cell–free immunity in Cecropia," Eur. J. Biochem., vol. 201, pp. 23–31 (1991).
Beck et al., "Invertebrate Cytokines III: Invertebrate Interleukin–1–like Molecules Stimulate Phagocytosis by Tunicate and Echinoderm Cells," Cellular Immunology, vol. 146, pp. 284–299 (1993).
Caput et al., "Identification of a common nucleotide sequence in the 3'–untranslated region of mRNA molecules specifying inflammatory mediators," Proc. Natl. Acad. Sci. USA, vol. 83, pp. 1670–1674 (1986).
Morvan et al., "In vitro activity of the antimicrobial peptide magainin 1 against *Bonamia ostreae*, the intrahemocytic parasite of the flat oyster *Ostrea edulis*," Mol. Mar. Biol., vol. 3, pp. 327–333 (1994).
Cooper et al., "Transgenic Catfish Expressing a Lytic Peptide Gene," in Research and Review, A Compilation of Abstracts of Research on Channel Catfish, Catfish Farmers of America (1995).
A. Miller, "Human Gene Therapy Comes of Age," Nature, vol. 357, pp. 455–460 (1992).
P. Klysten et al., "The cecropin locus in Drosophila; a Compact Gene Cluster Involved in the Response to Infection," E.M.B.O. J., vol. 9, pp. 217–224 (1990).
J. Reichhart et al., "Insect Immunity: Developmental and Inducible Activity of the Drosophila Diptericin Promoter," E.M.B.O. J. vol. 11, pp. 1469–1477 (1992).
Y. Tryselius et al., "CecC, a Cercropin Gene Expressed During Metamorphosis in Drosophila Pupae," Eur. J. Biochem., vol. 204, pp. 395–399 (1992).
G. Ciliberto et al., "Inducible and Tissue–Specific Expression of Human C–reactive Protein in Transgenic Mice," E.M.B.O. J., vol. 6, pp. 4017–4022 (1987).
Pelham et al., "DNA Sequences Required for Transcriptional Regulation of the *Drosophila* hsp70 Heat–Shock Gene in Monkey Cells and *Xenopus* Oocytes," in (Schlessinger et al., eds.) *Heat Shock From Bacteria ato Man*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 43–48 (1982).
U.S. application No. 07/069,653, Jaynes et al., filed Jul. 6, 1987.
U.S. application No. 07/102,175, Jaynes et al., filed Sep. 29, 1987.
U.S. application No. 07/336,181, Jaynes et al., filed Apr. 10, 1989.
E. Cameron et al. Br. Vet. J. 150: 9–24 (1994).
R. Guyonard et al. Biochemie 71: 857–63 (1989).
T. Chen. Transgenic Models in Med & Agric. Wiley–Liss (1990) pp. 127–139.

Primary Examiner—Bruce R. Campbell
Attorney, Agent, or Firm—John H. Runnels

[57] **ABSTRACT**

Novel means have been discovered for increasing the resistance of an animal host (including humans) to diseases caused by intracellular bacteria, protozoa, and viruses. The infection treated may, for example, be equine infectious anemia, or infection by the human immunodeficiency virus. Novel means have also been found for treating tumors. Augmentation of the host's defenses against infectious diseases or tumors is achieved by "arming" the host's cells with an exogenous gene encoding a natural or synthetic lytic peptide. For example, the transfection of hematopoietic stem cells and embryonic cells will produce animals with enhanced disease resistance; and transfection of TIL (tumor infiltrating lymphocytes) cells or other cells can be used in the treatment of tumors. Genes coding for a cecropin or other native or synthetic lytic peptide can be transferred and stably expressed in mammalian, bony fish, other vertebrate, and other animal cells. The transformed cells have the ability to produce and secrete a broad spectrum chemotherapeutic agent that has a systemic effect on certain pathogens, particularly pathogens that might otherwise evade or overcome host defenses.

3 Claims, No Drawings

TRANSGENIC FISH CAPABLE OF EXPRESSING EXOGENOUS LYTIC PEPTIDES

This is a continuation-in-part of International Application No. PCT/US94/07456, international filing date Jun. 30, 1994; which in turn is a continuation-in-part of two prior United States patent applications. (1) U.S. patent application Ser. No. 08/085,746, filed Jun. 30, 1993, now abandoned; and (2) U.S. patent application Ser. No. 08/084,879, filed Jun. 30, 1993, now abandoned.

The development of this invention was partially funded by the Government under grants 90364-5137 and 903-64-3105 awarded by the Department of Agriculture. The Government may have certain rights in this invention.

This invention pertains to transgenic fish and transgenic fish cells capable of expressing exogenous lytic peptides.

Lytic Peptides

Few effective treatments exist for either acute or chronic intracellular bacterial, protozoal, or viral diseases of animals, including humans. In many such infections, the infectious agent is localized within host cells. Due to the intracellular location of the infectious agents, the host immune system is often ineffective. Likewise, antipathogenic compounds are often ineffective, due to their difficulty in crossing host cell membranes.

Beck et al., "Invertebrate Cytokines III: Invertebrate Interleukin-1-like Molecules Stimulate Phagocytosis by Tunicate and Echinoderm Cells," Cellular Immunology, vol. 146, pp. 284–299 (1993) discusses relationships among phagocytotic mechanisms of different phyla.

Lytic peptides are small, basic proteins. Native lytic peptides appear to be major components of the antimicrobial defense systems of a number of animal species, including those of insects, amphibians, and mammals. They typically comprise 23–39 amino acids, and have the potential for forming amphipathic alpha-helices. See Boman et al., "Humoral immunity in Cecropia pupae," Curr. Top. Microbiol. Immunol. vol. 94/95, pp. 75–91 (1981); Boman et al., "Cell-free immunity in insects," Annu. Rev. Microbiol., vol. 41, pp. 103–126 (1987); Zasloff, "Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," Proc. Natl. Acad. Sci. USA, vol. 84, pp. 3628–3632 (1987); Ganz et al., "Defensins natural peptide antibiotics of human neutrophils," J. Clin. Invest., vol. 76, pp. 1427–1435 (1985); and Lee et al., "Antibacterial peptides from pig intestine: isolation of a mammalian cecropin," Proc. Natl. Acad. Sci. USA, vol. 86, pp. 9159–9162 (1989).

Known amino acid sequences for lytic peptides may be modified to create new peptides that would also be expected to have lytic activity by substitutions of amino acid residues that preserve the amphipathic nature of the peptides (e.g., replacing a polar residue with another polar residue, or a non-polar residue with another non-polar residue, etc.); by substitutions that preserve the charge distribution (e.g., replacing an acidic residue with another acidic residue, or a basic residue with another basic residue, etc.); or by lengthening or shortening the amino acid sequence while preserving its amphipathic character or its charge distribution. Lytic peptides and their sequences are disclosed in Yamada et al., "Production of recombinant sarcotoxin IA in Bombyx mori cells," Biochem. J., Vol. 272, pp. 633–666 (1990); Taniai et al., "Isolation and nucleotide sequence of cecropin B cDNA clones from the silkworm, Bombyx mori." Biochimica Et Biophysica Acta, Vol. 1132, pp. 203–206 (1992); Boman et al., "Antibacterial and antimalarial properties of peptides

that are cecropin-melittin hybrids," Febs Letters, Vol. 259, pp. 103–106 (1989); Tessier et al., "Enhanced secretion from insect cells of a foreign protein fused to the honeybee melittin signal peptide," Gene, Vol. 98, pp. 177–183 (1991); Blondelle et al., "Hemolytic and antimicrobial activities of the twenty-four individual omission analogs of melittin," Biochemistry, Vol. 30, pp. 4671–4678 (1991); Andreu et al., "Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity," Febs Letters, Vol. 296, pp. 190–194 (1992); Macias et al., "Bactericidal activity of magainin 2: use of lipopolysaccharide mutants," Can. J. Microbiol., Vol. 36, pp. 582–584 (1990); Rana et al., "Interactions between magainin-2 and Salmonella typhimurium outer membranes: effect of Lipopolysaccharide structure," Biochemistry, Vol. 30, pp. 5858–5866 (1991); Diamond et al., "Airway epithelial cells are the site of expression of a mammalian antimicrobial peptide gene," Proc. Natl. Acad. Sci. USA, Vol. 90, pp. 4596ff(1993); Selsted et al., "Purification, primary structures and antibacterial activities of β-defensins, a new family of antimicrobial peptides from bovine neutrophils," J. Biol. Chem., Vol. 268, pp. 6641 ff(1993); Tang et al., "Characterization of the disulfide motif in BNBD-12, an antimicrobial β-defensin peptide from bovine neutrophils," J. Biol. Chem., Vol. 268, pp. 6649 ff (1993); Lehrer et al., Blood, Vol. 76, pp. 2169–2181 (1990); Ganz et al., Sem. Resp. Infect. I., pp. 107–117 (1986); Kagan et al., Proc. Natl. Acad. Sci. USA, Vol. 87, pp. 210–214 (1990); Wade et al., Proc. Natl. Acad. Sci. USA, Vol. 87, pp. 4761–4765 (1990); and Romeo et al., J. Biol. Chem., Vol. 263, pp. 9573–9575 (1988).

Lytic peptides typically have a broad spectrum of activity (e.g., against gram negative bacteria, fungi, protozoa, and viruses). Their activity is both direct and indirect (e.g., virus-infected cells are destroyed, disrupting virus production) Thus some pathogens that have developed the ability to avoid host defenses are nevertheless susceptible to destruction by lytic peptides.

At least four families of naturally-occurring lytic peptides have been discovered in the last decade: cecropins, defensins, sarcotoxins, and magainins. Boman and coworkers in Sweden performed the original work on the humoral defense system of *Hyalophora cecropia*, the giant silk moth, to protect itself from bacterial infection. See Hultmark et al., "Insect immunity. Purification of three inducible bactericidal proteins from hemolymph of immunized pupae of *Hyalophora cecropia*," Eur. J. Biochem., vol. 106, pp. 7–16 (1980); and Hultmark et al., "Insect immunity. Isolation and structure of cecropin D. and four minor antibacterial components from cecropia pupae," Eur. J. Biochem., vol. 127, pp. 207–217 (1982).

Infection in *H. cecropia* induces the synthesis of specialized proteins capable of disrupting bacterial cell membranes, resulting in lysis and cell death. Among these specialized proteins are those known collectively as cecropins. The principal cecropins—cecropin A, cecropin B, and cecropin D—are small, highly homologous, basic peptides. In collaboration with Merrifield, Boman's group showed that the amino-terminal half of the various cecropins contains a sequence that will form an amphipathic alpha-helix. Andrequ et al., "N-terminal analogues of cecropin A: synthesis, antibacterial activity, and conformational properties," Biochem., vol. 24, pp. 1683–1688 (1985). The carboxy-terminal half of the peptide comprises a hydrophobic tail. See also Boman et al., "Cell-free immunity in Cecropia," Eur. J. Biochem., vol. 201, pp. 23–31 (1991).

Recently, a cecropin-like peptide has been isolated from porcine intestine. Lee et al., "Antibacterial peptides from pig

intestine: isolation of a mammalian cecropin," Proc. Natl. Acad. Sci. USA, vol. 86, pp. 9159–9162 (1989).

Cecropin peptides have been observed to kill a number of animal pathogens other than bacteria. See Jaynes et al., "In Vitro Cytocidal Effect of Novel Lytic Peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*," FASEB, 2878–2883 (1988); Arrowood et al., "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum*," J. Protozool., vol. 38, No. 6, pp. 161S–163S (1991); and Arrowood et al., "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum*," Antimicrob. Agents Chemother., vol. 35, pp. 224–227 (1991). However, normal mammalian cells do not appear to be adversely affected by cecropins, even at high concentrations. See Jaynes et al., "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," Peptide Research, vol. 2, No. 2, pp. 1–5 (1989); and Reed et al., "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide, " Mol. Reprod. Devel., vol. 31, No. 2, pp. 106–113 (1992).

Defensins, originally found in mammals, are small peptides containing six to eight cysteine residues. Ganz et al., "Defensins natural peptide antibiotics of human neutrophils," J. Clin. Invest., vol. 76, pp. 1427–1435 (1985). Extracts from normal human neutrophils contain three defensin peptides: human neutrophil peptides HNP-1, HNP-2, and HNP-3. Defensin peptides have also been described in insects and higher plants. Dimarcq et al., "Insect immunity: expression of the two major inducible antibacterial peptides, defensin and diptericin, in *Phormia terranvae*," EMBO J., vol. 9, pp. 2507–2515 (1990); Fisher et al., Proc. Natl. Acad. Sci. USA, vol. 84, pp. 3628–3632 (1987).

Slightly larger peptides called sarcotoxins have been purified from the fleshfly *Sarcophaga peregrina*. Okada et al., "Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of *Sarcophaga peregrina* (flesh fly) larvae," J. Biol. Chem., vol. 260, pp. 7174–7177 (1985). Although highly divergent from the cecropins and defensins, the sarcotoxins presumably have a similar antibiotic function.

Other lytic peptides have recently been found in amphibians. Gibson and collaborators isolated two peptides from the African clawed frog, *Xenopus laevis*, peptides which they named PGS and $Gly^{10}Lys^{22}$PGS. Gibson et al., "Novel peptide fragments originating from PGL_α and the caervlein and xenopsin precursors from *Xenopus laevis*," J. Biol. Chem., vol. 261, pp. 5341–5349 (1986); and Givannini et al., "Biosynthesis and degradation of peptides derived from *Xenopus laevis* prohormones," Biochem. J., vol. 243, pp. 113–120 (1987). Zasloff showed that the Xenopus-derived peptides have antimicrobial activity, and renamed them magainins. Zasloff, "Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial DNA sequence of a precursor," Proc. Natl. Acad. Sci. USA, vol. 84, pp. 3628–3632 (1987).

Syntbesis of nonhomologous analogs of different classes of lytic peptides has been reported to reveal that a positively charged, amphipathic sequence containing at least 20 amino acids appeared to be a requirement for lytic activity. Shiba et al., "Structure-activity relationship of Lepidopteran, a self-defense peptide of Bombyx more," Tetrahedron, vol. 44, No. 3, pp.787–803 (1988); and unpublished data from our laboratory. The published literature has reported that a 20-mer appeared to possess roughly the minimum alpha-helix length needed to span a cell membrane. Smaller peptides (or lower concentrations of peptide) not only failed to kill cells, but were reported actually to stimulate cell growth. Reed et al., "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," Mol. Reprod. Devel., vol. 31, No. 2, pp. 106–113 (1992).

Cecropins have been shown to target pathogens or compromised cells selectively, without affecting normal host cells. The synthetic lytic peptide known as Shiva 1 has been shown to destroy intracellular *Brucella abortus-*, *Trypanosoma cruzi-*, *Cryptosporidium parvum-*, and infectious bovine herpes virus 1 (IBR)-infected host cells, with little or no toxic effects on noninfected mammalian cells. See Jaynes et al., "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," Peptide Research, vol. 2, No. 2, pp. 1–5 (1989); Wood et al., "Toxicity of a Novel Antimicrobial Agent to Cattle and Hamster cells In vitro," Proc. Ann. Amer. Soc. Anim. Sci., Utah State University, Logan, UT. J. Anim. Sci. (Suppl. 1), vol. 65, p. 380 (1987); Arrowood et al., "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum,*" J. Protozool., vol. 38, No. 6, pp. 161S–163S (1991); Arrowood et al., "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum,*" Antimicrob. Agents Chemother., vol. 35, pp. 224–227 (1991); and Reed et al., "Enhanced in vitro growth of murine fibroblast cells and preimplantation embryos cultured in medium supplemented with an amphipathic peptide," Mol. Reprod. Devel., vol. 31, No. 2, pp. 106–113 (1992).

Morvan et al., "In vitro activity of the antimicrobial peptide magainin 1 against *Bonamia ostreae*, the intrahemocytic parasite of the flat oyster *Ostrea edulis,*" Mol. Mar. Biol., vol. 3, pp. 327–333 (1994) reports the in vitro use of a magainin to selectively reduce the viability of the parasite *Bonamia ostreae* at doses that did not affect cells of the flat oyster *Ostrea edulis*.

Also of interest are the following commonly-assigned patent applications: Jaynes et al., "Method for Introduction of Disease and Pest Resistance Into Plants and Novel Genes Incorporated Into Plants Which Code Therefor," United States patent application Ser. No. 07/373,623, filed Jun. 29, 1989 abandoned; Jaynes et al., "Plants Genetically Enhanced for Disease Resistance," United States patent application Ser. No. 07/646,449, filed Jan. 25, 1991 abandoned; Jaynes et al., "Therapeutic Antimicrobial Polypeptides, Their Use and Methods for Preparation," Ser. No. 07/069,653, filed Jul. 6, 1987 abandoned; Jaynes et al., "Inhibition of Eucaryotic Pathogens and Neoplasms and Stimulation of Fibroblasts and Lymphocytes with Lytic Peptides," United States patent application Ser. No. 07/102, 175, filed Sep. 29, 1987 abandoned; Jaynes, "Lytic Peptides: Their Use in the Treatment of Microbial Infections, Cancer and in the Promotion of Growth," United States patent application Ser. No. 07/336,181, filed Apr. 10, 1989 abandoned; and McLaughlin et al., "Amphipathic Peptides," United States patent application Ser. No. 08/232,525, filed Apr. 22, 1994 abandoned.

It is believed (without wishing to be bound by this theory) that lytic peptides act by disrupting cell membranes, and that normal host cells protect themselves through their ability to repair the resulting membrane damage. By contrast, bacteria, protozoa, and compromised host cells are unable (or less able) to repair damaged membranes. Because parasitized cells have a diminished capacity to repair membranes, after a lytic peptide "attack" they are preferentially destroyed, while adjacent normal cells repair their membranes and survive.

At least three modes have been proposed for the lytic peptide-membrane interaction that leads to cytolysis: (1)

The amphipathic helix is located on the membrane surface, and the presence of the helix in the head group region disorders the lipid bilayer. Dawson et al., "The interaction of bee melittin with lipid bilayer membranes," Biochem. Biophys. Acta., vol. 510, pp. 75–86 (1978). (2) Peptide oligomers form ion channels in the membrane, resulting in osmotically-induced lysis. Tosteson et al., "The sting—melittin forms channels in lipid bilayers," Biophys. J., vol. 36, pp. 109–116 (1981). (3) The lytic peptide causes aggregation of native membrane proteins, resulting in the formation of channels or pores. Burt et al., "Role of membrane proteins in monosodium urate crystal-membrane interactions. I. Effect of pretreatment of erythrocyte neuraminidase," J. Rheumatol., vol. 17, pp. 1353–1358 (1990).

Many intracellular obligate pathogens live inside host cells because they are vulnerable to host defenses when outside the cell, where they may be destroyed by humoral or cellular defenses, or by conventional therapeutic agents. Also, known viruses and intracellular protozoa require a staging development within a host cell before becoming infectious; if released prematurely they will not be infective. Evidence indicates that the released infective stages in bacterial, fungal, and protozoal pathogens are directly destroyed by lytic peptides. See Jaynes et al., "In vitro effect of lytic peptides on normal and transformed mammalian cell lines," Peptide Research, vol. 2, No. 2, pp. 1–5 (1989); Jaynes et al., "In Vitro Cytocidal Effect of Novel Lytic Peptides on *Plasmodium falciparum* and *Trypanosoma cruzi*," FASEB, 2878–2883 (1988); Arrowood et al., "Hemolytic properties of lytic peptides active against the sporozoites of *Cryptosporidium parvum,*" J. Protozool., vol. 38, No. 6, pp. 161S–163S (1991); and Arrowood et al., "In vitro activities of lytic peptides against the sporozoites of *Cryptosporidium parvum,*" Antimicrob. Agents Chemother., vol. 35, pp. 224–227 (1991).

Plants Transformed with Lytic Peptide Genes

A number of synthetic lytic peptides have been synthesized, retaining some properties of native lytic peptides. For example, Shiva 1 was designed as a substitution analog of native Cecropin B, having 46% homology to the natural Cecropin B molecule. However, the hydrophobic properties and charge density of the native structure were conserved in the synthetic peptide. Data supporting the ability of the Shiva I gene to enhance disease resistance has been obtained from transgenic plants. Genes encoding synthetic lytic peptides were chemically synthesized and cloned into the binary vector pBI121. For the less active peptide SB-37 (a cecropin analog), expression was controlled by a constitutive promoter, the 35S cauliflower mosaic virus 5' region-nopaline synthetase-3' polyadenylation cassette (Rogers et al. "Improved vectors for plant transformation expression cassette vectors and new selectable markers," Meth. Enz. vol. 153, pp. 253–305 (1987)). For the more active Shiva 1, expression was controlled by the wound-inducible plant promoter for proteinase inhibitor II ("Pill") (Sanchez-Serrano et al., "Wound-induced expression of a potato proteinase inhibitor II gene in transgenic tobacco plants," EMBO J. vol. 6, pp. 303–306 (1987); Jaynes et al., "Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum,*" Plant Science (1993).

In non-wounded potato plants, Pill accumulates in the tubers, with non-detectable levels of the protein in leaves, stem or roots. When the leaves are wounded, however, expression of the gene is induced not only in the wounded leaves, but also in non-wounded upper and lower leaves and in the upper part of the stem. Pena-Cortes et al., "Systemic induction of proteinase-inhibitor-II gene expression into potato plants by wounding," Planta, vol. 174, pp. 84–91 (1988).

Transgenic tobacco plants with genes coding for lytic peptides have also been obtained via Agrobacterium transformation. Bioassays testing the disease resistance of F_1 progeny indicated that, compared to transgenic controls and SB-37 plants, Shiva-containing tobacco seedlings exhibited delayed wilt symptoms and reduced disease severity and mortality after infection with a highly virulent strain of *Pseudomonas solanacearum.* (*P. solanacearum* is a vascular pathogen that causes severe wilting.) Jaynes et al., "Expression of a cecropin B lytic peptide analog in transgenic tobacco confers enhanced resistance to bacterial wilt caused by *Pseudomonas solanacearum,*" Plant Science (1993). No enhanced resistance was observed for the plants producing the synthetic peptide SB-37, presumably because of its low bioactivity against this pathogen. Destefano-Beltran et al., Mol. Biol. of the Potato, pp. 205–221 (1990).

In contrast to this work in plants, to the knowledge of the inventors, no previous work has resulted in the successful expression of exogenous cecropin (or any other lytic peptide) in mammalian or other animal cells. In fact, very few invertebrate genes have ever been stably expressed in a vertebrate cell, and even fewer non-mammalian genes have ever been stably expressed in a mammalian cell.

Transformation of Eukaryotic Genomes

A major problem in transforming DNA into eukaryotic cells, especially into the cells of mammals and other vertebrates, is the stable integration of the exogenous DNA into a recipient chromosome. Several techniques are currently used for the delivery of the DNA into the recipient host, techniques that have shown varying degrees of success. The first, and currently most common, method for transformation of animals is the microinjection of exogenous DNA into a one- or two-cell embryo. This procedure has several drawbacks, including the following difficulties: (1) the technique requires a level of skill that is not available in most laboratories; (2) the procedure is very time-consuming, often requiring an entire day to microinject a few hundred embryos; and (3) the method has a relatively low success rate—typically about 1–3% of the injected embryos are observed to have a stable insertion.

The second most common procedure is electroporation. Electroporation has advantages over microinjection, primarily in speed. Using electroporation, several thousand embryos can be transformed in a day. Major limitations include the following: (1) the availability of embryos, (2) means to maintain and screen the embryos and (3) the approximately 50% lethality levels caused by the electric currents typically used. The high lethality is somewhat offset by the increased number of embryos demonstrating positive expression, a number that can approach 15%, about five times that of the best microinjection rates.

A third method, currently gaining popularity, is the use of lipofection to deliver DNA packaged inside liposomes. Lipofection has the advantage over electroporation that it is not as lethal to the cells. Lipofecton typically results in a 1–2% increase in successful transformations as compared to electroporation.

However, each of these three transformation methods shares the common disadvantage of relying on homologous recombination of the target DNA into recipient chromosomal DNA; the necessary homologies may not always exist, and even where they do exist, the recombination

events may be slow. In addition, each of the above percentages for successful DNA expression is decreased by a factor of approximately two if the only insertions counted are those that are successfully passed on to subsequent generations of cells or offspring.

Because the limiting factor for most transformations is typically the availability of embryos, it is desirable to optimize the chances of stable DNA insertion into the available embryos. An embryo, regardless of source, should be in the 2–4 cell stage of development to maximize the probability of a stable insertion that will be incorporated into the germ cells. Obtaining embryos at this stage requires careful timing; and a quick response time will often be necessary to ensure that the cells are manipulated while in the proper stage, before their development progresses too far.

Current procedures for the genetic transformation of higher organisms are not only time-consuming, but are also expensive in terms of person-hours used. For these and other reasons, there remains an unfilled need for a more efficient means of delivering and stably integrating exogenous DNA into the chromosomes of a higher organism.

Transposons

A transposon is a mobile genetic element capable of inserting at random into a DNA sequence. See generally Lewin, Genes IV, pp. 649–671 (1990); and Lewin, Genes V, pp. 999–1031 (1994). Most prior research on transposons has been conducted with bacteria; a limited number of studies have been conducted with transposons in eukaryotic cells. A wild-type transposon typically includes a gene encoding a transposase (an enzyme controlling transposition), flanked by two sequences called insertion sequences. It may also carry other genes, such as a gene for antibiotic resistance. The insertion sequences are generally inverted repeats of one another (exact or closely related). Wild-type transposons typically insert preferentially in certain regions or "hot spots" in the host genome.

Prior work with transposons has used transposons for creating mutations, e.g., disrupting existing genes; and in studying pathogenesis. In such applications, unmodified wild-type transposons have several disadvantages. These disadvantages include undesired homologous recombinations resulting from the relatively large size of the insertion sequences, and spontaneous transposition of a transposon to another location in the genome induced by the transposase. Spontaneous transposition is relatively rare, but it can cause the expression of a new phenotypic trait, making interpretation of results difficult.

"Mini-transposons" have recently been developed to attempt to circumvent some of the problems associated with wild-type transposons. Kleckner et al., "Uses of Transposons with Emphasis on Tn10," pp. 139–180 in Miller (ed.), *Methods in Enzymology*, vol. 204 (1991). These mini-transposons have been modified from the wild-type transposons in three ways: (1) The transposase gene has been removed from its native site between the two insertion sequences, and instead placed upstream from the transposon. This rearrangement promotes a more stable insertion that should not move following insertion, because the transposase gene is lost upon insertion. (2) The insertion sequences have been shortened to about 70 base pairs in length, compared to sequences that are typically well over 100 base pairs in wild-type transposons. This shorter length greatly inhibits unwanted homologous recombinations. (3) The entire transposon "cassette" has been placed under the control of an inducible promoter, such as the ptac promoter. The ptac promoter is only read in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), allowing complete control over when the transposase is activated to cause transposition of the transposon. Furthermore, the transposase is often mutated so that it is less specific to "hot spots," sequences where the wild-type transposon preferentially inserts. This lowered specificity increases the rate of insertion into the genome.

Mini-transposons have previously been used in place of wild-type transposons to cause mutations by disrupting genes, and to study pathogenesis. It has not previously been suggested that a mini-transposon might be used as a vector for stably transforming an exogenous gene into a eukaryotic chromosome.

THE INVENTION

Novel means have been discovered for increasing the resistance of a mammalian (including human), vertebrate, or invertebrate host to diseases caused by intracellular bacteria, protozoa, and viruses The infection treated may, for example, be equine infectious anemia, or infection by the human immunodeficiency virus. Novel means have also been found for treating tumors.

Augmentation of the host's defenses against infectious diseases or tumors is achieved by "arming" the host's cells with an exogenous gene. The host's own leukocytes, other cells involved in resistance to infection, or other cells are transformed with a gene conferring the ability to synthesize and secrete natural or synthetic lytic peptides, such as native cecropin B or synthetic lytic peptides such as SB-37, Shiva I through X, or Manitou. The expression of the genes is induced when needed to combat pathogens.

For example, the transfection of hematopoietic stem cells and embryonic cells will produce animals (or humans) with enhanced disease resistance; and transfection of leukocytes such as TIL (tumor infiltrating lymphocytes) cells, neutrophils, macrophages, or cytotoxic lymphocytes can be used in the treatment of tumors. Stable incorporation of the gene into an egg, a zygote, an early-stage embryo, or a totipotent embryonal stem cell will cause the gene to be carried in germ cells, and thus to be inherited by future generations. Such a transformed animal (e.g., a chicken or a catfish) would have economic significance.

Transgenic animals that have been or that will be engineered with genes and promoters in accordance with the present invention include domesticated donkeys, horses, cows, sheep, goats, pigs, chickens, and turkeys; as well as various species that are raised or that may be raised in whole or in part through aquaculture, including bony fish, crustaceans, and bivalves such as the following: channel catfish, koi, red drum, hybrid striped bass, trout sp., salmon sp., shrimp sp., lobster sp., crawfish spa, crab sp., and oyster sp.

It has been discovered that genes coding for a cecropin or other native or synthetic lytic peptide, such as native cecropin B or Shiva 1, can be transferred and stably expressed in mammalian, other vertebrate, and other animal cells. The transformed cells have the ability to produce and secrete a broad spectrum chemotherapeutic agent that has a systemic effect on certain pathogens, particularly pathogens that might otherwise evade or overcome host defenses. The peptide's expression is preferably induced only in areas of infection, where it will most effectively augment the host's defense systems. Animal cells, including mammalian cells and fish cells, have been transfected with cecropin B, and will also be transfected with cecropin analogs. It has been observed that expression of the exogenous gene does not damage a healthy recipient cell.

Novel means have also been discovered for transforming a eukaryotic cell with a gene under the control of an exogenous promoter that is responsive to an inducer of an acute-phase peptide or protein. As an example, it was unexpected that the transfer of a gene coding for an invertebrate lytic peptide, or a synthetic homolog of such a peptide, can enhance the immune potential of a vertebrate animal. It is particularly unexpected that placing (or leaving) the gene under the control of a native invertebrate promoter such as the native *Hyalophora cecropia* promoter leads to appropriately induced expression in vertebrate cells. To our knowledge, the stable insertion and expression of a gene in a vertebrate cell under the control of an invertebrate promoter have never previously been accomplished. To our knowledge, the stable insertion and expression of a gene in a eukaryotic cell under the control of an exogenous promoter responsive to an inducer of an acute phase protein has never previously been accomplished.

A novel transposon-based vector has also been developed, a novel vector that enhances the integration of DNA into a host genome, particularly a eukaryotic genome. The novel vector has been used, for example, in the transformation of mammalian and fish cells with a gene coding for the lytic peptide cecropin B. The novel vector allows the rapid and efficient transformation of a eukaryotic genome. Its use does not require the high level of skill needed for microinjections. Nor does it rely on homologous recombination events for a successful transformation, as do the prior methods of microinjection, electroporation, and lipofection.

These and other advantages were achieved by constructing a novel modification of mini-transposons to adapt them to carry a gene of interest into a genome. Such an adaptation of mini-transposons has never previously been suggested.

Briefly, the novel vector for transforming an exogenous gene into a eukaryotic cell comprises: a gene encoding a transposase; two transposon insertion sequences; an exogenous gene, where the exogenous gene is located between the two insertion sequences; and a promoter that is adapted to cause the transcription of the transposase, where one of the insertion sequences is located between the transposase gene and the exogenous gene; and where the transposase is adapted to excise from the vector a fragment comprising the two insertion sequences and the portion of the vector between the two insertion sequences, and to insert the excised fragment into a chromosome of a eukaryotic cell.

This arrangement insures that the transposase gene is not incorporated into the target chromosome, insuring that the transformation will be stable. Descendants of a cell transformed with the vector will not have a copy of the transposase gene. Without a transposase gene to encode a transposase, there will be nothing to promote excision of the exogenous gene from the genome.

The examples given below use a vector based on the Tn10 transposon, but analogous vectors constructed from other transposons will work in the present invention as well. Examples of transposons are known in the art, and include, for example, AC7, Tn5SEQ1, Tn916, Tn951, Tn1721, Tn2410, Tn1681, Tn1, Tn2, Tn3, Tn4, Tn5, Tn6, Tn9, Tn30, Tn101, Tn903, Tn501, Tn1000(γδ), Tn1681, Tn2901, AC transposons, Mp transposons, Spm transposons, En transposons, Dotted transposons, Mu transposons, Ds transposons, dSpm transposons, and I transposons.

All transposons function in an essentially similar manner, so the transposon used to construct novel vectors in accordance with this invention is a matter of choice. The insertion sequences of a transposon will recognize their target sequences (typically 8–10 base paris in length), regardless of the species in whose genome the target sequences are found; and they will insert into the target sequence the DNA lying between the flanking insertion sequences. Target sequences for a given transposon will occur in a genome at a statistically predictable frequency, such that it is statistically likely that any given eukaryotic genome will have multiple target sequences for the insertion sequences from any given transposon.

General

Studies in our laboratory have shown that a variety of treatments make cells susceptible to at least some lytic peptides, in situations where corresponding untreated cells are resistant to the peptides. Resistant cells can be made susceptible by treatment with cytoskeletal inhibitors, cytochalasin D and colchicine, or by chilling the cells to 4° C. for 15 minutes prior to exposure to peptide. Resistant cells treated with trypsin also became extremely susceptible to lysis by the lytic peptides. A common factor in each of these examples of induced susceptibility appears to be an altered plasma membrane and/or cytoskeleton. The alteration may interfere with the repair of damaged membrane by hampering endocytosis or exocytosis.

Further evidence of the selective susceptibility of macrophages to the lytic peptides was obtained in a series of experiments using mouse peritoneal macrophages and *Listeria monocytogenes*, an obligate intracellular, gram-positive bacterium. Normal, non-activated, resident peritoneal macrophages (R1); and activated macrophages derived from the peritoneal cavities of Listeria-immune mice inoculated intra-peritonally 17 hours earlier with Listeria (L1) were exposed in vitro to Listeria and then treated with a lytic peptide. Additionally, resident macrophages from normal non-Listeria immune mice were infected in vitro with Listeria (L2) and treated with the Shiva I peptide. The Shiva peptide had little effect on control resident macrophages (RI). Neither Listeria alone nor the peptide alone (without Listeria infection) resulted in significant cell death. However, Listeria-infected macrophages (L1 and L2) were killed by the peptide. Macrophages from Listeria-immune mice (L1) re-exposed to Listeria by intraperitoneal inoculation 17 hours earlier were killed when exposed to the peptide. The presence of intracellular Listeria was confirmed by microscopic examination of the groups of cells prior to treatment.

As described in greater detail below, we have developed a construct carrying the gene for the native cecropin peptide and the native cecropin promoter, a construct that can be inducibly expressed in animal cells, and that has been successfully expressed in mammalian cells and fish cells. This result is quite unexpected, particularly because a native insect promoter has been successfully used to regulate expression of a gene in mammalian cells. The construct was made so that the native gene, or synthetic genes for analogues of the native peptides, can be placed under the control of the native cecropin promoter.

The plasmid construct designated "PCEP" carries both the native cecropin promoter and the native cecropin gene. Electroporation of the pCEP construct into fetal donkey dermal cells ("FDD cells") resulted in the expression of antibacterial substances when those cells were co-cultured with viable *E. coli*. This antibacterial activity was not observed in control electroporated FDD cells.

Transformation of Fetal Donkey Dermal Cells

Fetal donkey dermal cells were chosen as a model system for cecropin expression. This cell line was chosen for several

reasons. First, it was known from prior studies that these cells are resistant to lysis by lytic peptides. Second, this cell line had previously been used to study the antiviral activity of several lytic peptides against Equine Infectious Anemia (EIA) infection. The EIA-infected cells were lysed by the peptides, while uninfected cells were not. The cell line has been demonstrated to be refractory to damage from electroporation. Finally, these cells will act as hosts in vitro for *Listeria monocytogenes* and *Trypanosoma cruzi*, agents to be used to evaluate the antibacterial and anti-protozoal activity of cells expressing native or analog lytic peptides.

FDD cells were cultivated in Eagle's minimum essential medium (MEM), supplemented with Earle's salts, L-glutamine, nonessential amino acids, 5% fetal bovine serum (FBS), and the antibiotics penicillin (100 unit/ml) and streptomycin (100 μg/ml). Cells were split once a week until the desired number were obtained. Conditioned medium that had been clarified from a freshly split culture of FDD cells after 24 hours of culture was used to maintain the cells after electroporation.

Prior to electroporation, FDD cells were rinsed with phosphate buffered saline (PBS), scraped from the flask, rinsed again with PBS, and counted. The concentration of cells was adjusted to 9×10^5 cells/100 μL, and the cells were placed in a BioRad 0.4 cm (electrode gap width) electroporation cuvette. To this cuvette were also added 400 μL of PBS and 1.4 μg of linearized pCEP DNA. The cuvette and its contents were kept on ice until electroporation. The cells were electroporated at 2.0 KV and 1 μF in the presence of 10 mM IPTG. Immediately after electroporation, 0.5 ml of conditioned medium was added to the cells, which were then incubated on ice for a 10 min recovery period. The cells were then transferred to flasks containing equal parts of conditioned medium and fresh medium, and were allowed to form a monolayer. Monolayered cells were trypsinized, subpassaged in 24-well plates, and allowed to form a monolayer. These cells were then subpassaged into 96-well plates. In the 96-well plates, the cells were grown without any antibiotics, and allowed to form a monolayer.

A two-step method was used to enrich for the population of cells expressing the antibacterial substance. It has been observed that antibacterial activity in cells expressing lytic peptides is associated with a loss of cellular sensitivity to trypsin. This trait allowed the selective removal of cells not expressing the antibacterial substance. Cultures were first exposed to *E. coli*, followed by exposure to trypsin. Cultures demonstrating antibacterial activity were scraped to remove the trypsin-resistant cells. These trypsin-resistant cells were then further diluted and subcultured. Those cells continued to divide to produce a monolayer culture of FDD cells resistant to bacterial colonization. Cultures of cells expressing antibacterial activity were demonstrated to contain the cecropin gene by Southern blotting. Electroporated cell monolayers unable to prevent bacterial colonization were presumed to be negative for expression of the cecropin gene.

Pathogen Challenges

When the monolayer was complete, a first challenge with pathogenic *E. coli* (isolated from a case of equine cystitis) was added at a concentration of 10 bacteria/well. This low concentration was chosen to attempt to stimulate cecropin production, without overwhelming the culture. In the second challenge, bacteria were added at a rate of 1000/well and incubated overnight. After incubation, the wells were examined for colonization of the bacteria in clumps on the surface of the FDD cell monolayer. Bacterial colonization ranged from wells with no bacterial colonies to wells overgrown with bacterial colonies. Wells in which there was no colonization or only slight colonization (about 15% of the total) were rinsed, and antibiotic medium was added back to the wells. Cells were harvested and transferred to flasks to allow monolayer formation. It was observed that the FDD cultures that prevented colonization of bacteria also showed a loss of trypsin sensitivity. This same phenotypic trait had previously been observed in FDD cells following exposure to exogenous cecropin analogs.

The transformed FDD cells expressed the cecropin gene inductively, rather than constitutively. When the cells were split before exposure to bacteria, they were susceptible to treatment with trypsin; but after exposure to bacteria and subsequent cecropin production, the cells were resistant to trypsinization.

FDD cells positive and negative for antibacterial activity were expanded in 75 cm^2 flasks Both groups of cells were challenged with 10^3–10^5 EIA viral particles and incubated at 37° C. Daily examination of the cells showed the negative control cells acting as normally-infected EIA cells. However, the FDD cells positive for antibacterial activity demonstrated an increased cytopathic effect, manifested approximately 3 days before that of the control cells. This increased cytopathic effect is believed to be due to cecropin production by the FDD cells, a hypothesis that will be tested through a series of deletion mutations, as described below. These results demonstrate the usefulness of the present invention in treating virally-infected cells.

Electroporation of FDD cells has been repeated five times, and bacterial challenges have been performed on all five groups. Cells positive for antibacterial activity have been detected in each of the groups. Cells from the earliest electroporations have been passaged numerous times; they have also been frozen and brought back to culture; all without any apparent loss in viability or phenotypic changes. The incorporation of DNA appeared to be stable: cecropin mRNA was detected in cells descended from the first electroporation that had subsequently been passaged four times.

Confirmation of Transformation by Southern Blot

Southern blot analysis was performed both on FDD cells that were positive for antibacterial activity, and on FDD cells that were negative for antibacterial activity. Electroporated cells not receiving DNA were used as negative controls in the Southern analysis. The chromosomal DNA was harvested from FDD cells using the protocol of Ausubel et al., Current Protocols in Molecular Biology, vols. 1 and 2 (1991) for tissue-culture cells, and that DNA was electrophoresed on a 0.8% agarose gel. The DNA was transferred from the gel to a positively charged nylon membrane (Zeta Probe GT, Bio-Rad Laboratories, Richmond, Calif.), where it was probed with the cecropin gene isolated from pMON 200. The probe was prepared, and the subsequent hybridization was performed, using the non-isotopic Genius 1™ nonradioactive DNA labelling and detection kit (Boehringer Mannheim Corporation, Indianapolis, Ind.). The high stringency protocol was performed according to the manufacturer's instructions. Positive hybridization results were observed only in the electroporated cells receiving pCEP DNA, and in the pCEP vector used as a positive control No hybridization was seen in the electroporated cells that did not receive pCEP DNA.

Confirmation of Transformation by PCR

FDD cells positive for cecropin were rinsed with PBS and fed with MEM that contained antibiotics as described above. The positive clones were passaged three times to try to

insure that no cecropin or associated mRNA remained in the cells. After the third passage formed a monolayer, the cells were split into two groups. One group was challenged with bacteria, and the second group received a PBS treatment without bacteria as described above. After a 24-hour incubation, both groups were harvested, and RNA was harvested by the method of Ausubel et al., Current Protocols in Molecular Biology, vols. 1 and 2 (1991). Briefly, 3.5 ml of 4 M guanidinium thiocyanate solution was added per each 10^6 cells, both to lyse the cells and to inactivate any RNase present. The resulting lysate was suspended in 5.7 M cesium chloride, and centrifuged at 150,000× g for 16 hours to separate RNA from DNA. The RNA pellet was resuspended in TES (10 mM TrisHCl, pH 7.4; 5 mM EDTA; 1% sodium dodecyl sulfate (SDS)), 3 M sodium acetate, and 100% ethanol, and then placed on dry ice/ethanol for 30 min to precipitate the RNA. The pelleted RNA was resuspended in 200 μL of sterile, distilled water, and quantitated by measuring absorbance at 260 nm and 280 nm.

The poly(A)-RNA (i.e., mRNA) was then separated from the tRNA and rRNA as follows. Total RNA was denatured by heating to 70° C. for 10 minutes to expose any poly(A)+ sites, and to disrupt secondary structures. The RNA mixture was passed through an oligo(dT) column to bind the poly (A)+ sites. The column was then washed twice to remove rRNA and tRNA, and then 2 mM EDTA/0.1% SDS was used to elute the mRNA. The mRNA was precipitated with ethanol and sodium acetate, and resuspended in TE (10 mM TrisHCl, pH 8.0, and 1 mM EDTA).

The mRNA was then used for PCR amplification using the semi-quantitative protocol of Dallman et al., "Semi-quantitative PCR for the analysis of gene expression," in Rickwood et at. (eds.), PCR: *A Practical Approach* (1991). Briefly, synthesis of cDNA from the mRNA was performed with reverse transcriptase from Moloney murine leukemia virus (Gibco-BRL). Using primers to the preceropin gene sequence, the cDNA was then amplified via PCR: cycle at 94° C., 1 min (denaturing); at 55° C., 2 min (annealing); and at 72° C. 1 min (extension). After 10–20 cycles, 15 μL samples can be taken at the end of every 5th cycle, and stored in 96-well microtiter plates. Quantitation of the unknown cDNA was achieved by having internal oligonucleotide standards that were titrated against the cDNA. The concentration of the standard at which the amount of amplification product was equal to the amount of amplification product from the target approximated the starting concentration of the experimental DNA (to within, say, an order of magnitude).

One group of transformed FDD cells was challenged with bacteria for 6 hours, after which the cells were harvested for mRNA isolation. Purified mRNA was reverse-transcribed to cDNA using Moloney Murine virus reverse transcriptase. Following the procedure described above, the CDNA was added to a PCR-amplification mixture with primers to pre-cecropin B, and was cycled 30 times in the thermocycler. FDD cells without vector DNA were used as negative controls. The mRNA from one group of cells showing antibacterial activity had a 180 bp fragment that corresponded to the size that was expected to be amplified, based on the design of the primers This band was not present in the control cell mRNA, nor in the non-challenged cecropin-transformed FDD cells, showing that the cecropin was not constitutively produced.

Acute Phase Response Mechanisms

Our results demonstrate the ability of FDD cells to recognize the native cecropin promoter, and to express the cecropin B peptide in response to exposure to pathogenic *E. coli*. Replication of the cells was not affected. More generally, we expect that a wide variety of animal cells will express genes placed under the control of exogenous promoters responsive to inducers of acute-phase proteins or peptides, due to the high degree of homology many such proteins and peptides have maintained across widely-separated taxa.

Without wishing to be bound by this theory, the fact that the insect promoter was appropriately induced in a mammalian cell suggests that there is substantial homology between the acute phase response (APR) mechanisms of insect cells and mammalian cells—sufficient homology that the mammalian cells recognize the insect promoter and express the gene controlled by that promoter.

Various APR's from various species that have been identified to date share certain similarities at the genetic level, similarities that may be related to their transcription. Known IL-1 sequences, tumor necrosis factor (TNF), human lymphotoxin (LT), human and mouse granulocyte-macrophage colony stimulating factors (GM-CSF), and fibronectin sequences have a common 8 base sequence—TTATTTAT—in the region of the gene that is transcribed into an untranslated portion of the 3' end of the mRNA. Although the function of this sequence is not known, it is believed to influence the termination codon in some fashion; in homologous molecules in different organisms, e.g., human and mouse IL-1, the distance to the termination codon is conserved. Alternatively, it may serve as a possible target for endoribonucleases involved in the rapid removal of mRNA when inflammation ceases. Caput et al., "Identification of a common nucleotide sequence in the 3'-untranslated region of mRNA molecules specifying inflammatory mediators," Proc. Natl. Acad. Sci. USA, vol. 83, pp. 1670–1674 (1986); Toniatti et al., "Regulation of the Human C-reactive Protein Gene, a major marker of Inflammation and Cancer," Mol. Biol. Med., vol. 7, pp. 199–212 (1990). This conserved octamer has recently been identified in the gene for the LPS-binding protein of the American cockroach, *Periplantea americana*. Jomori et al., "Molecular Cloning of cDNA for Lipopolysaccharide binding Protein from the Hemolymph of the American Cockroach, *Periplaneta americana*," J. Biol. Chem. vol. 266, pp. 13318–13323 (1991).

Another common APR sequence in the portion of the gene corresponding to the 3' untranslated region of the mRNA's appears to be the sequence ATTTA, a sequence that is believed to be responsible for unstable mRNA. Jomori et al., "Molecular Cloning of cDNA for Lipopolysaccharide binding Protein from the Hemolymph of the American Cockroach, *Periplaneta americana*," J. Biol. Chem. vol. 266, pp. 13318–13323 (1991). Also identified as conserved regions in many APR's are gene sequences corresponding to non-translated sequences in the 5' end of the mRNA's. One such sequence is TGGRAA, which has been reported for α2-macroglobulin, α1-acid glycoprotein, γ-fibrinogen, haptoglobin and human C-reactive protein (CRP). Dente et al., "Structure of the Human α_1-acid glycoprotein gene: sequence homology with other human acute phase protein genes," Nucl. Acids Res., vol. 13, pp. 3941–3952 (1985); Fey et al., "Regulation of rat liver Acute-Phase genes by Interleukin-6 and production of hepatocyte stimulating factors by rat hepatoma cells," Ann. N.Y. Acad. Sci. vol. 557, pp. 317–331 (1989); Toniatti et al., "Regulation of the Human C-reactive Protein Gene, a major marker of Inflammation and Cancer," Mol. Biol. Med. vol. 7, pp. 199–212 (1990). Other conserved 5' transcription factors include the

CCAAT/enhancer binding protein (C/EBP) and the CCATT box and enhancer core sequences which are common in a wide range of APR associated gene sequences. Kaling et al., "Liver-Specific Gene Expression: A-Activator-Binding Site, a Promoter Module Present in Vitellogen and Acute-Phase Genes," Mol. Cell. Biol., vol. 11, pp. 93–101 (1991).

Conserved APR sequences for other molecules were compared to Xanthopoulas' published sequence for the native cecropin. Xanthopoulas et al., "The structure of the gene for cecropin B, an antibacterial immune protein from *Hyalophora cecropia,*" Eur. J. Biochem., vol. 172, pp. 371–376 (1988) Though not described by Xanthopoulos et al., several sequences were found corresponding to conserved sequences for other APR's. The first conserved sequence found was in the 3' untranslated region—TTATTTAT—found at positions 814–821 and at 972–979, and found again at position 848–855 with only one base substitution. The conserved sequence ATTTA corresponding to unstable mRNA is found in the 3' untranslated region a total of six times. In the 5' untranslated region of the cecropin gene, the sequence TGGRAA is found twice with one base substitution in each sequence, but whether this sequence is functional remains to be determined. A conserved sequence corresponding to the CCATT box and to the enhancer core is found two times in the 5' untranslated region of the gene. With the benefit of hindsight, these homologies make plausible our discovery that the native cecropin B promoter and peptide gene respond to at least some vertebrate APR inducers to elicit expression in vertebrate cells.

It is believed (without wishing to be bound by this theory) that an as-yet-unidentified cytokine is responsible for eliciting both cecropin production and secretion. Unpublished data from our laboratory suggests that cecropin is produced in the moth in response to the presence of a cytokine, Injection of the pupal stage of the giant silkworm moth with either *E. coli* or *E. coli* LPS (lipopolysaccharide or endotoxin), or with human recombinant interleukin-1 resulted in hypertrophic changes in the fat body of the pupae, and in elevated levels of cecropin in the hemolymph. Without wishing to be bound by this theory, it is believed that a cytokine or similar inducing agent is responsible for inducing expression of the gene in vertebrate cells as well when the native moth promoter is used to control the gene.

The Cecropin Promoter

The function of the cecropin promoter will be studied by inserting the neo-CAT gene (chloramphenicol acetyl transferase, a reporter gene) under its control in place of the lytic peptide gene. Transformed cells will be screened by assaying for CAT enzyme activity. The neo-CAT gene was chosen because it will allow pre-selection of FDD cells containing the CAT gene by selecting in the presence of neomycin. Once a transformed cell line with the CAT gene is established, cecropin promoter activity will be studied by altering the promoter with point and/or deletion mutations in the conserved promoter binding sequences, and assaying for altered CAT expression. The neo-CAT gene will be digested from the vector pMAMneo-CAT (Clontech, Palo Alto, Calif.) and inserted into the Sal I site of pCEP to replace the cecropin peptide gene. The conserved 5' APR signals in the cecropin promoter will be altered by oligo-directed mutagenesis as described by Zoler et al., Methods Enzymol., vol. 100, pp. 468–500 (1983). Briefly, ssDNA from construct 5'A-94 will be used as a template for the in vitro synthesis of the complementary strand The mutations are designed to insert into a Bal I restriction cite. A set of synthetic oligonucleotides carrying the base substitution(s) are used as primers for the second strand. The mutant cecropin promoter is then cloned into the Bal I site of pCEP linked to the CAT gene. Using this approach, substitutions will be created in the conserved CCAATT, TTGGACA, and TTGGAAC sequences of the promoter.

Electroporation will be performed as described earlier, and enzyme production will be screened by SDS-PAGE and Western blot analysis with rabbit anti-CAT. The intact cecropin gene (including the unaltered promoter) will be run simultaneously in all assays for comparison. Identification of the antibacterial substance as cecropin B, and its localization within FDD cells and on their surface, will be accomplished using rabbit anti-cecropin B polyclonal antibodies with an indirect fluorescent antibody procedure.

The intact cecropin B gene and the neo-CAT gene in pCEP will be run concurrently in the following experiments to probe the means of expression. Cecropin- and neo-CAT-producing FDD cells will be prepared as described above using the bacterial, LPS, or IL-1 challenge method. Cells positive for cecropin and neo-CAT will be rinsed with PBS and cultured in MEM containing antibiotics. The positive clones will be passaged three times to insure that no cecropin, CAT enzyme, or RNA's associated with their production are left in the cells. After each third passage forms a monolayer, the cells will be split into two groups; one group will be challenged with bacteria in PBS, and the second group will receive a PBS treatment without bacteria. After a 24-hour incubation, the RNA from both groups will be harvested as described by the method of Ausubel et al., Current Protocols in Molecular Biology, vols. 1 and 2 (1991), as described above.

The mRNA is then used for PCR amplification using the semi-quantitative protocol previously described, using primers to the pre-cecropin and CAT gene sequences. If, as expected, cecropin and CAT expression is induced, then only cells exposed to the bacteria or cytokine will yield positive results for the mRNA. If, however, either protein is constitutively produced, then both the negative controls and the "induced" cells will demonstrate a positive response for mRNA; and if this is the case the quantitative portion of this experiment will determine whether the levels increase in response to the inducing agent.

To understand how the cell is induced to produce cecropin, responses to various agents will be measured. The same experiments will be conducted on cells containing vectors in which the cecropin promoter has been altered. In the unlikely event that the cecropin promoter is determined not to be the promoter responsible for cecropin production, then the primers made to the cecropin and/or CAT genes will be used to sequence into the FDD genome to identify and characterize the active promoter. Purified LPS (Sigma Chemical) will be added to cells negative for cecropin production; the supernatant will be collected; and the LPS will be removed with neutralizing antibody (anti-LPS). Four groups of cells will then be exposed to different inducing agents: (1) LPS-induced-cell supernatant treated with anti-LPS and polymyxin B to remove LPS; (2) IL-1 added to the FDD cells; (3) FDD cells induced with whole bacteria; and (4) FDD cells receiving a PBS placebo as a control. The supernatant will be collected from each group and split into two groups: half of the supernatant will be used to determine cecropin activity on *E. coli* or CAT activity, and the other half will be quantitated for the amount of cecropin or CAT production using a polyclonal anti-cecropin or anti-CAT. Some of the supernatant from the LPS-induced group and the IL-1-induced group will be treated with anti-IL-1 and used to challenge cecropin-producing cells. Cecropin or

CAT production will be measured using the bacterial assay and the polyclonal antibody to cecropin or CAT.

If LPS alone is responsible for inducing cecropin expression, then only the LPS and bacteria-induced FDD cells should show a positive cecropin or CAT response. If IL-1 is responsible for induction, then all of the cells except the control should show a positive response, and this response should be neutralized with anti-IL-1, which would then yield a negative result for cecropin or CAT when the supernatant is used to induce FDD cells. Other cytokines (e.g., IL-6, TNF (Biogen), IL-2 (Bioferon), and purified γ-IFN (Bioferon)) will be assayed in a similar fashion.

Other Promoters

Promoters other than the native cecropin promoter are suitable for use in the present invention. Such promoters include other native promoters for native lytic peptides, and other native promoters that are responsive to APR inducers. The identification and isolation of such promoters is within the ability of one of ordinary skill in the art, given the teachings of the present specification, including particularly the following discussion.

Where such promoters are not already identified in the literature, they may be identified as follows. With a lytic peptide, the DNA upstream from the coding region of the gene may be sequenced, and the portion comprising the promoter identified through standard means, by searching for conserved sequences that are typical of promoters.

APR inducers, such as IL-1, IL-2, IL-6, C-reactive protein, LPS binding protein, lymphotoxin, granulocyte-macrophage colony stimulating factors, fibronectin, interferons, and tumor necrosis factor, induce other genes as well. Genes induced by an APR inducer may be identified through means known in the art, including isolation of the proteins thus translated, or identification of induced mRNA's through subtractive hybridization. Where the protein is isolated, through means known in the art: it may be partially sequenced; a probe for a genomic library or (preferably) for an appropriate cDNA library may be prepared; and the cloned gene may then be sequenced, including its promoter region.

Construction of Plasmid pCEP, and Transfection of *E. coli* with pCEP

The plasmid pCEP is a pBR322 derivative that carries the gene for ampicillin resistance and the ColE1 origin of replication, but in which a segment from base pair 105 to base pair 2345 has been deleted to streamline the plasmid, to allow incorporation of the native cecropin promoter and gene, or other gene of interest. The cecropin gene segment is the 5.9 Kb fragment isolated from the vector pMON 200 by a restriction digest with EcoR I and Xho I. Both ends were filled to create a blunt-ended fragment, as were the ends of the pBR322 plasmid.

The native cecropin gene was ligated into the modified pBR322 vector to give a construct of 9.3 Kb, using a modification of Ausubel et al., Current Protocols in Molecular Biology, vols. 1 and 2 (1991); and Sambrook et al., *Molecular Cloning, A Laboratory Manual, 2nd* ed. (1989). The modification, discussed further below, increases the chance of blunt-end fragment insertion into the plasmid. The resulting recombinant plasmid, designated pCEP, was then electroporated into *Escherichia coli* NM554 (Stratagene, La Jolla, Calif.) using a BioRad Gene Pulser™ electroporator, under conditions described by the manufacturer for *E. coli.* Electroporated bacteria were plated onto Brain-Heart Infusion (BHI) agar containing 50 μL/ml of ampicillin, and incubated at 37° C. overnight. Because the ptac promoter is not read unless induced, the potentially lethal lytic peptide gene may be maintained in *E. coli* without the danger that the peptide will kill the *E. coli.*

It is preferred that the promoter controlling the transcription of the transposase be inducible, so that the transposase gene is not transcribed until an inducing stimulus (e.g., IPTG for the ptac promoter) is supplied. However, it would also be possible to use a constitutive promoter for the transposase, particularly a promoter that is inactive in *E. coli* or other prokaryotic host, but that is expressed constitutively in a eukaryotic cell. The vector would be lost or diluted following a number of cell divisions, so that continuing transpositions of the inserted segment should not occur.

Colonies growing on the BHI/ampicillin plates were subcultured in BHI/ampicillin broth for plasmid screening (Qiagen, Chatsworth, Calif.) and freezing at −70° C. Plasmid preparations of the isolates were examined by agarose gel electrophoresis. A 1% gel was electrophoresed for 4 hours at 4 V/cm, stained with 0.4 μg/ml ethidium bromide for 10 min., and destained in distilled water for 30 min. A supercoiled plasmid DNA ladder (Sigma Chemical) was used as a DNA size reference; bands corresponding to ~9.3 Kb were removed from the agarose and purified using the Ehuqnick™ DNA Purification Kit protocol. Purified plasmid DNA was then electroporated back into competent *E. coli* NM554 and selected on BHI/ampicillin plates. Because *E. coli* NM554 is a plasmid-less strain, this additional purification-electroporation step insured that there was only one plasmid type per cell by eliminating pBR322—pBR322 self-ligated dimers.

Confirmation of the pCEP plasmid was obtained by growing *E. coli* NM554 on BHI/ampicillin plates; making plasmid preparations; making restriction digests with BamH I to yield two fragments of 2.3 and 7.0 Kb; and Southern blotting of the restriction digest under very stringent conditions using the cecropin gene isolated from pMON 200 as a probe.

A sample of this transformed *E. coli* strain NM554 containing plasmid pCEP with the cecropin gene was deposited with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Md. 20852 on Jun. 30, 1993, and was assigned ATCC Accession No. 69342. This deposit was made pursuant to a contract between ATCC and the assignee of this patent application, Board of Supervisors of Louisiana State University and Agricultural and Mechanical College. The contract with ATCC provides for the permanent and unrestricted availability of the progeny of this *E. coli* strain to the public on the issuance of the U.S. patent describing and identifying the deposit or the publication or the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progeny of this *E. coli* strain to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto under pertinent statutes and regulations. The assignee of the present application has agreed that if the *E. coli* strain on deposit should die or be lost or destroyed when cultivated under suitable conditions, it will be promptly replaced on notification with a viable culture of the same *E. coli* strain. As the term is used in the claims below, it should be understood that the "pCEP" plasmid encompasses not only the specific plasmid included in this ATCC deposit, but also any plasmid that is substantially identical to that plasmid, with the specific exception that the gene placed under the control of the cecropin promoter may vary; it is intended that the term "pCEP" plasmid should encompass any such plasmid, regardless of the specific DNA inserted.

After confirmation of the pCEP plasmid was completed, a large scale plasmid isolation was performed. A 250 ml

culture of the transformed *E. coli* was grown in BHI/ampicillin broth on a rotary shaker at 37° C. until an absorbance at 600 nm of A_{600}=0.4 was obtained, at which time chloramphenicol was added to give a final concentration of 180 μg/ml before amplifying the plasmid. Shaking was continued overnight. After 24 hours, the pCEP DNA was harvested using the Qiagen plasmid isolation protocol (Qiagen, Chatsworth, Calif.) and column to obtain pure, high quantity (0.7 μg/μL) plasmid DNA. A restriction digest using Pst I was conducted on 20 μg of the pCEP DNA to linearize the DNA prior to electroporation into a mammalian cell line as described above. The enzyme Pst I was chosen because it linearizes the plasmid without cutting the cecropin gene.

The pCEP plasmid itself was constructed as follows. Plasmid pNK2859 containing Tn10 derivative 103 (obtained from Dr. Nancy Kleckner, Department of Biochemistry and Molecular Biology, Harvard University; see Kleckner et al., "Uses of Transposons with Emphasis on Tn10," pp. 139–180 in Miller (ed.), *Methods in Enzymology*, vol. 204 (1991)) was digested with the enzyme BamH I. This digestion had the effect of removing the kanamycin antibiotic resistance marker from the transposon, but leaving the insertion sequences flanking the kanamycin resistance gene intact. The digest resulted in two bands approximately 3.2 Kb and 1.6 Kb in size. A double digestion was performed on the pMON 200 vector with the enzymes EcoR I and Xho I, yielding fragments of approximately 6 Kb and 9.7 Kb. These two enzymes remove the native cecropin B gene intact from the pMON 200 vector in the 6 Kb fragment. The resulting fragments from the two digests were separated by agarose gel electrophoresis on a 1% gel run at 40 V for four hours. The 3.2 Kb fragment from the transposon vector, and the 6 Kb fragment from the pMON 200 vector were excised from the gels, and each was separated from the agarose using the Eluquick™ DNA Purification Kit (Schleicher and Schuell, Keene, N.H.). This method minimized DNA shearing, and allowed efficient recovery of the desired fragments.

The purified DNA fragments did not have complimentary ends, so a blunt-end ligation protocol was designed, based on modifications of Ausubel et al., Current Protocols in Molecular Biology, vols. 1 and 2 (1991); and Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed. (1989). Briefly, blunt end fragments were created using Klenow fragment and dNTP's (final concentration of 0.5 mM) at an incubation temperature of 30° C. for 15 min, followed by deactivation at 75° C. for 10 min. Both the cecropin fragment and the transposon vector fragment were extracted in phenol:chloroform; precipitated in isopropanol; and resuspended in 10 μL of TE buffer (10 mM Tris, 5 mM EDTA, pH 8.0). The cecropin fragment was then ligated onto the transposon vector fragment using T4 DNA ligase and 40% PEG (polyethylene glycol) according to Sambrook et al., *Molecular Cloning, A Laboratory Manual*, 2nd ed. (1989). PEG was used to enhance the ligation of blunt-ended fragments, and to minimize the formation of concatemers of the same DNA.

The ligation mixture was incubated overnight at 20° C. The following day DNA was extracted in phenol:chloroform; precipitated in isopropanol; and resuspended in 10 μL of sterile, distilled H_2O. *E. coli* NM554 (Stratagene, La Jolla, Calif.) was made competent for electroporation using the protocol of BioRad, Inc (BioRad Laboratories, Hercules, Calif.). Electroporation was conducted at 1.65 KV, 200 Ω, and 25 μF using 10^{10} cells of competent *E. coli*. Two electroporations were performed: (1) 10^{10} cells with 5 μL of the ligated cecropin/vector DNA, and (2) a negative control using 10^{10} cells with 5 μL of sterile, distilled H_2O. Each electroporation mixture was immediately placed in 1 ml of BHI (Brain Heart Infusion, Difco Inc.) broth at 37° C. for one hour to recover, and to begin expression of the ampicillin resistance marker. After the one-hour recovery period, 200 μL of each mixture was applied to an agar plate using the spread plate technique to cover the entire plate. *E. coli* receiving the ligated pCEP plasmid were plated onto BHI agar plates supplemented with 100 μg/ml of ampicillin, while the *E. coli* receiving the distilled H_2O were plated both on BHI agar plates with ampicillin, and on BHI agar plates without ampicillin as controls. All plates were incubated overnight at 37° C. All colonies growing on the BHI/ampicillin agar plates were then grown in BHI/ampicillin broth, and frozen at −70° C.

Each colony was also screened for the presence of a plasmid of approximately 9.391 Kb: 5.99 Kb from the cecropin gene segment, plus 3.401 Kb from the vector DNA. When suitable candidates were identified, the potential pCEP-bearing *E. coli* were propagated in a 100 ml culture of BHI/ampicillin broth grown to an absorbance of A_{600}=0.4, at which time chloramphenicol was added to a final concentration of 170 μg/ml. The cultures were allowed to continue shaking overnight at 37° C. for plasmid replication. The pCEP DNA was then harvested using a modified version of the Qiagen (Qiagen, Chatsworth, Calif.) protocol. Insertion of the cecropin B gene was confirmed using Southern blot analysis, with labelled cecropin gene as a probe. The nonisotopic Genius 1™ nonradioactive DNA labelling and detection Kit (Boehringer Mannheim) was used to perform the Southern analysis, and each step was conducted under stringent hybridization conditions. Isolates positive for the cecropin insert were then tested for the production of cecropin B as described above.

Other constructs will also be made. The first of these constructs is a streamlined native cecropin promoter-cecropin B peptide vector. The gene sequence obtained from the pMON 200 vector is rather cumbersome to work with, in that it is 6 Kb long. Using the published sequence and restriction map of cecropin B (Xanthopoulas et al., "The structure of the gene for cecropin B, an antibacterial immune protein from *Hyalophora cecropia*," Eur. J. Biochem., vol. 172, pp. 371–376 (1988)) a construct will be made that includes the entire gene, and that is about 3.1 Kb, about half the size of the 6 Kb sequence. DNA from the pMON 200 vector will be partially digested with EcoR I and BamH I. The fragments will be separated on an agarose gel, and purified with the Eluquick™ DNA Purification Kit protocol described above. These fragments will then be cloned into a pBR322derived plasmid with polylinkers with either EcoR I- or BamH I-complementary ends to the digested fragment to insure proper orientation in the construct.

Streamlining the cecropin insert will allow easier removal of the gene encoding the native peptide, to facilitate replacement with a gene encoding a synthetic peptide such as Shiva I. If needed, another promoter can be substituted for the native cecropin promoter through means known in the art. At this time, however, the native cecropin promoter is preferred.

Creating a smaller delivery vehicle for the cecropin gene will also aid in sequencing the gene in a host cell using nucleic acid amplification techniques such as the polymerase chain reaction ("PCR") Primers can be synthesized that will amplify internal segments of the cecropin gene, or segments extending into the host genome, to determine both the orientation and location of the gene in the host chromosome. A smaller fragment will enable sequencing with less time and expense.

ANOTHER EXAMPLE

Disease-Resistant Catfish

Catfish farming has rapidly become a major agricultural industry in the southeastern United States, particularly in Alabama, Louisiana, Mississippi, Arkansas, and South Carolina. A major factor limiting the economic success and future growth of the catfish industry is disease. Bacterial disease is the primary cause of mortality in commercially-reared channel catfish (*Ictalurus punctatus*), accounting for 57.5% of 9575 total cases examined from 1987 to 1989 by diagnostic labs in Alabama, Mississippi, and Louisiana. Infectious diseases cause an estimated 10% annual mortality in an industry where each 1% loss to disease translates to about a 5.5% loss of profits. The catfish industry could be greatly enhanced if this 55% loss of profits could be reduced.

Vaccination is one possible mechanism for reducing disease caused by bacterial or other pathogens. Of the major bacterial diseases associated with commercially-raised channel catfish, *Edwardsiella ictaluri* is the leading cause of mortality, followed by *Cytophaga columnaris* and Aeromonas sp. *E. ictaluri* alone accounts for approximately 50% of the catfish disease cases submitted to aquatic animal diagnostic laboratories in the southeastern United States. Some progress has been made in live attenuated vaccine construction for *E. ictaluri* and *A. hydrophila*. Though a marketable vaccine may be available in the near future for *E.ictaluri*, a vaccine for *A. hydrophila* is many years away, and a live attenuated vaccine for *C. columnaris* may never be feasible due to the difficulty in obtaining viable, stable mutants capable of eliciting an immune response. There remains an unfilled need for improved, inexpensive methods of combatting bacterial and other disease in catfish.

An alternative to creating a vaccine for each pathogen plaguing the catfish industry is to create a transgenic strain of catfish having lymphocytes capable of destroying invading organisms before the disease process is established. Toward this end, we have transformed catfish eggs with the cecropin B gene, using the novel transposon-based pCEP vector.

Plasmid pCEP was replicated in large quantities by amplification in *E. coli* strain NM554. (The same plasmid in the same *E. coli* strain as was deposited with the American Type Culture Collection under accession number ATCC 69342.) Plasmid DNA from the amplified *E. coli* cultures was harvested with Qiagen Maxi-Columns (Qiagen Inc., Chatsworth, Calif.) using the manufacturer's recommended protocol. This procedure typically yielded 500–800 μg/ml of plasmid DNA from 250 ml broth culture. Purified pCEP DNA was linearized by Pst I restriction endonuclease digestion for 2 hours at 37° C. The reaction was stopped by the addition of 3 μl of 0.5 M EDTA, and holding at 70° C. for 10 min. The DNA was purified by phenol:chloroform extraction and isopropanol precipitation. Linearized DNA was resuspended to a concentration of 100 μg/ml in Hanks' balanced salt solution (HBSS), and was stored at 4° C. until it was used in electroporation.

Two healthy, mature, male channel catfish from a research stock maintained at Louisiana State University, Baton Rouge, La. were killed, and their testes were removed as a source for spermatozoa. Adherent tissue was dissected away, and the testes were blotted, dried, and weighed. Two grams of anterior testis from each fish were placed in a plastic bag, and were dissociated by crushing. The spermatozoa were suspended in 10 ml of HBSS, and were stored at 4° C. until used.

Five pairs of healthy, mature (2–4 kg, 50–80 cm long) channel catfish from a research stock maintained at Louisiana State University, Baton Rouge, La. were spawned using standard procedures. Female channel catfish were injected with a luteinizing hormone-releasing hormone analog, D-Ala^6 Des Gly^{10} LH-RH-ethyl amide (Peninsula Laboratories, Belmont, Calif.), at a dose of 100 μg/kg, and were paired with male channel catfish in 80 liter aquaria supplied with aerated, flow-through well water. The fish were monitored for spawning behavior. Once egg release had begun, the females were removed from the aquaria, anesthetized in 0.02% tricaine methanesulfonate, rinsed, and dried. Eggs were stripped by application of pressure along the abdomen.

Before fertilization, eggs were electroporated with the linearized pCEP DNA using a BioRad Gene Pulser™ electroporator (Richmond, Calif.). Eggs were rinsed in Hank's buffer and placed in a specially designed cuvette with 3 ml of DNA solution. Approximately 500 channel catfish eggs per treatment were placed in a silicon-coated electroporation chamber (6 cmx6 cmx6 cm). Eggs were either sham-electroporated (control), or were electroporated with 100 μg/ml of linearized pCEP plasmid DNA containing the cecropin gene. The field strength for the electroporation was 125 V/cm, with 200 Ω resistance, and 1 μF capacitance, with each treatment receiving either 1 or 3 pulses with a time constant of 7–10 msec, at a pulse interval of 1 sec.

The DNA solution was then removed, the eggs were rinsed in HBSS, and the electroporated eggs were then fertilized with the previously harvested spermatozoa using standard techniques. The eggs were allowed to water-harden for approximately 15 minutes. Once water-hardened, zygotes were treated with a pH-buffered 1.5% sodium sulfite solution to remove the gelatinous mass, and to inhibit possible fungal colonization. The sodium sulfite solution was then decanted from the zygotes, followed by a water rinse to remove excess sodium sulfite. Zygotes were then placed in hatching jars in a recirculating raceway with aeration, where they were maintained until hatching.

Five days after hatching, fish fry produced from different females and treatments were pooled into two groups: fish that had been electroporated with pCEP DNA, and fish that had been electroporated without exogenous DNA. Thirty-nine potentially transgenic fish, and forty-three sham-electroporated controls were acclimated for two weeks at 27° C. in 95-liter aquaria equipped with both under-gravel and outside filtration.

Six-week-old fry were then challenged with a high dose, 8×10^5 cfu/ml (final concentration), of a known virulent strain of *E. ictaluri* by adding the culture to the water. The fry were allowed to swim in the inoculum for 2 hours with aeration, but no filtration. No subsequent water exchanges were conducted, although normal filtration resumed after two hours. Fish were observed three times daily for signs of the enteric septicemia typically caused by *E. ictaluri*. The posterior kidneys of dead or moribund fish were dissociated and used to establish cultures on 5% sheep blood agar plates The plates were incubated at 30° C., and the resulting colonies were screened by biochemical analysis and agarose gel electrophoresis to confirm the presence of *E. ictaluri* in the dead and moribund fish.

Transformed channel catfish were grown to approximately 15 cm in length, and blood samples were collected for assay by the polymerase chain reaction (PCR). Samples of genomic DNA were extracted both from the potentially transgenic channel catfish, and from the blood of control

sham-electroporated catfish by the guanidine hydrochloride method. Normal channel catfish DNA was also spiked with cecropin DNA as a positive control. To demonstrate that the PCR procedures were working in samples that were negative for the cecropin gene, as an additional positive control we used primers targeted to the C_H^4 exon from the channel catfish gene encoding the constant region of the immunoglobulin μ heavy chain. The PCR reactions were set up as follows: 10 μM dNTP's, 1x Taq buffer, 5 units of Taq polymerase, 1% DMSO, 2 mM $MgSO_4$, 0.26 μM of each PCR primer, and sterile distilled water to 100 μl. Two primers, AGACTTGACTCCGCTGCATAAGTG (SEQ ID NO. 1) and TACCGTTTCTGATGTTGCGACC (SEQ ID NO. 2), were designed to amplify an 846 bp segment from the genomic cecropin DNA. (These two primers do not anneal to cecropin cDNA.) The reaction conditions were 95° C. for 2 min to denature the template, followed by 35 cycles programmed as follows: 95° C. for 30 sec, 52° C. for 30 sec, and 72° C. for 60 sec. After PCR, the samples were electrophoresed for 2 hours at 6.0 V/cm in a 2% agarose gel containing 0.3 μg/ml ethidium bromide. Size marker ΦX174, digested with HaeIII (Sigma Chemical Co., St. Louis, Mo.), was used to generate DNA size references. The banding patterns were observed on a MacroVue ultraviolet transilluminator (Pharmacia LKB Biotechnology, Piscataway, N.J.), and were photographed (Photoman PHH Series, Hoefer Scientific Instruments, San Francisco, Calif.).

To determine whether the cecropin gene was transcribed, mRNA was isolated and reverse-transcribed to cDNA for PCR analysis. To test the inducibility of the cecropin gene in response to an invading pathogen, blood samples were taken prior to challenge from channel catfish known to be positive for the cecropin gene and from channel catfish that were siblings, but that had not received the transgene. Then to induce cecropin production, all catfish (transgenic and controls) were challenged by intramuscular injection with $8x10^8$ irradiated *E. ictaluri*. Blood samples for mRNA isolation were taken 14 hours after the challenge. Blood was collected from the caudal vessel with a 3-ml syringe and a 22 gauge needle containing 150 μl ACD solution (Becton-Dickinson, vacutainer #6406) per ml of blood as an anticoagulant. After samples were collected, they were immediately placed in 1-ml cryovials and placed in liquid nitrogen until mRNA isolation.

The mRNA was isolated using the Oligotex™ RNA Isolation reagents and columns (Qiagen, Inc., Chatsworth, Calif.), and reverse-transcribed to cDNA using standard methods. The polymerase chain reaction for the cDNA samples was conducted as described above, except as otherwise described in this paragraph. For the template DNA, a 5 μl aliquot of the cDNA was used. The primer sequences TTTTCTTCGTGTTCGCTTTGGTTCTG (SEQ ID NO. 3) and ATCGCCGGTCCAGCCTTGACAATAC (SEQ ID NO. 4) were designed to amplify a 135-bp fragment of the cecropin cDNA. These two primers will also amplify a 649-bp fragment of genomic cecropin DNA. As a control, PCR on the plasmid vector was also run, yielding a 649-bp fragment. The annealing temperature was 54° C. for 30 sec. The PCR products were electrophoresed on a 3% agarose gel for 2 hours at 6.0 V/cm, along with the ΦX174/Hae III reference size markers. Samples were optimized with the Invitrogen™ PCR Optimizer kit, using the manufacturer's protocol with 1 unit of Taq polymerase.

The control channel catfish challenged with *E. ictaluri* demonstrated symptoms of septicemia beginning 10 days after immersion challenge, and began dying on day 11. By day 14, twenty-six of forty-three control fish were dead (60.4% mortality). The potentially transgenic fish did not show signs of septicemia or mortality until day 30. By day 33, thirteen of thirty-nine (33% mortality) of the potentially transgenic fish were dead. Compared to the control, mortality in the experimental group was substantially lower, and occurred substantially later. The mortality among the experimental group is attributed to toxic shock from the abnormally high concentrations of *E. ictaluri* used in this experiment. Future experiments are planned at lower concentrations of pathogen to test this hypothesis. The survivors from this experiment were used in the PCR experiments to detect presence or absence of the cecropin gene.

With the genomic-targeted primers (SEQ ID NOS. 1 and 2), PCR was expected to amplify an 846-bp fragment from the genomic cecropin gene. The values calculated by linear regression for both the positive control and the positive bands for the channel catfish on the agarose gel were 820 bp, within limits of experimental error. Of 18 potentially transgenic catfish assayed for the presence of the cecropin B gene, 9 (or 50%) yielded positive bands for that gene, while none of the 10 negative control fish assayed to date had such a band. All samples, both control and experimental, were positive for the heavy chain immunoglobulin exon, demonstrating that the PCR procedure worked properly.

The primers targeted to cecropin cDNA will yield a 135 bp fragment following PCR amplification. The same primers will amplify a 649 bp fragment when used to assay genomic DNA, the difference lying in the excision of an intron. These expected 649 bp and 135 bp bands were in fact observed following PCR amplification of genomic DNA and cDNA, respectively, from the challenged experimental fish. These bands were absent from all the controls, including the pre-challenge mRNA/cDNA from the blood of the same fish that later tested positive for cecropin mRNA/cDNA after *E. ictaluri* challenge, demonstrating that transcription of the cecropin mRNA was induced by the pathogen challenge.

The data clearly demonstrated that the cecropin B gene was stably inserted into the genome of channel catfish with the pCEP vector at high efficiency, and that the gene is inductively expressed in response to an important catfish pathogen, *Edwardsiella ictaluri*. Experiments to detect cecropin B mRNA from blood drawn from the same catfish will be performed using standard techniques known in the art (see, e.g., the procedures described above to detect mRNA from FDD cells). These experiments are expected to demonstrate the expression of the cecropin B gene in the transformed catfish.

FURTHER EXAMPLES

Making Transgenic Bony Fish via Lipofection, with Channel Catfish and Koi as Exemplary Embodiments

A drawback to working with many species of fish, including channel catfish, is the length of time required for the fish to reach sexual maturity—typically 3–4 years for channel catfish. When transgenic catfish are generated by electroporating eggs as described above, a minimum of three years must therefore elapse before the heritability of the transgene can be established with certainty. While we have every reason to believe that the transgene will be transmitted to offspring, heritability cannot be established with certainty until actual offspring can be examined.

Lipofection provides an alternative method of creating transgenic broodstock (probably mosaics), at least a portion

of whose gametes will contain the transgene. If lipofection is performed before the gonads recrudesce in spring (i.e., preferably in late winter), then natural spawning can take place, and the heritability of the transgene can be evaluated more quickly. In many applications, production of transgenic fish by lipofection may also be more economical than electroporation.

Lipofection uses a cationic lipid to deliver DNA to cells. The liposome is a unilamellar lipid membrane with both polar and nonpolar faces. When liposomes are mixed with DNA in aqueous solution, essentially all the DNA is encapsulated by the polar side of the lipid membranes, leaving the nonpolar surface exposed to the exterior. The nonpolar surface later interacts and fuses with nonpolar cell membranes to deliver DNA into cells, whether the cells be in cell culture, or in vivo.

As an initial demonstration of the lipofection technique, sexually immature catfish were used, because smaller fish are easier to handle than larger fish. Twenty specific pathogen free channel catfish (catfish that had never been exposed to *E. ictaluri*) were obtained from the Aquatic Pathobiology Laboratory at the School of Veterinary Medicine, Louisiana State University, Baton Rouge, La. Five fish per treatment were acclimated at 26° C. for two weeks in 35 liter aquaria equipped with both under-gravel and outside filtration. Once acclimated, these fish were used for lipofection.

Lipofectin™ reagent (Gibco BRL, Gaithersburg, Md.) was used per the manufacturer's instructions to encapsulate the linearized pCEP DNA. Briefly, 15 μL of Lipofectin™ reagent and 2.0 μg of linearized pCEP DNA were mixed together, and then incubated for 15 min. Following incubation, the following intraperitoneal injections were made into the channel catfish:

Group 1, 15 μL Lipofectin™/2.0 μg pCEP complex in 100 μL 0.85% NaCl solution +0.5 mL IPTG

Group 2, 2.0 μg pCEP in 100 μL 0.85% NaCl solution +0.5 mL IPTG

Group 3, 2.0 μg pCEP in 100 μL 0.85% NaCl solution

Group 4, 15 μL Lipofectin™ in 100 μL 0.85% NaCl solution

Intraperitoneal injections were made because the gonads and hematopoietic organs are exposed to the cavity, giving the liposome/DNA complex direct access to the organs for fusion to occur.

The fish were held for ten days, and then 0.1 mL blood in sodium citrate was withdrawn per fish from the caudal vein. DNA was extracted from the blood using the QiaAmp™ Blood Kit (Qiagen Inc., Chatsworth Calif.). PCR was conducted on each sample as described previously, using as primers SEQ ID NOS. 1 and 2, and the reaction products were electrophoresed on a 2% agarose gel. The pCEP vector DNA was used as a positive control in the PCR reactions.

All 5 Group 1 fish tested positive for the cecropin B gene, while none of the fish from Groups 2, 3, and 4 tested positive. It is expected that the cecropin B gene was incorporated into the gonads, and will be expressed in offspring Similar experiments will also be performed on sexually mature channel catfish, so that offspring may be tested more quickly.

We have also performed a pCEP lipofection on two sexually mature male Koi, following the lipofection procedure described above. Testing is currently underway to confirm that the cecropin B gene can be detected in sperm from these lipofected Koi, and that the cecropin B gene will be appropriately expressed inductively in the offspring of these Koi.

Miscellaneous

The complete disclosures of all references cited in this specification are hereby incorporated by reference, as are the complete disclosures of the three priority applications International Application No. PCT/US94/07456, international filing date Jun. 30, 1994; U.S. patent application Ser. No. 08/085,746, filed Jun. 30, 1993 abandoned; and U.S. patent application Ser. No. 08/084,879, filed Jun. 30, 1993 abandoned.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 4

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGACTTGACT CCGCTGCATA AGTG 24

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

-continued

(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TACCGTTTCT GATGTTGCGA CC 22

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTTTCTTCGT GTTCGCTTTG GTTCTG 26

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCGCCGGTC CAGCCTTGAC AATAC 25

We claim:

1. A transgenic catfish whose genome comprises a gene encoding cecropin B operably linked to the native cecropin B promoter, wherein said cecropin B promoter functions to direct expression of the cecropin B gene; and wherein the expression of said cecropin B gene imparts resistance to pathogenic bacteria.

2. A transgenic koi whose genome comprises a gene encoding cecropin B operably linked to the native cecropin B promoter, wherein said cecropin B promoter functions to direct expression of the cecropin B gene; and wherein the expression of said cecropin B gene imparts resistance to pathogenic bacteria.

3. A transgenic bony fish whose genome comprises a gene encoding cecropin B operably linked to the native cecropin B promoter, wherein said cecropin B promoter functions to direct expression of the cecropin B gene; and wherein the expression of said cecropin B gene imparts resistance to pathogenic bacteria.

* * * * *

EXHIBIT 2

US006307121B1

(12) **United States Patent**
Winn

(10) **Patent No.: US 6,307,121 B1**
(45) **Date of Patent: Oct. 23, 2001**

(54) **BACTERIOPHAGE-BASED TRANSGENIC FISH FOR MUTATION DETECTION**

(75) Inventor: **Richard N. Winn**, Athens, GA (US)

(73) Assignee: **The University of Georgia Research Foundation, Inc.**, Athens, GA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/322,725**

(22) Filed: **May 28, 1999**

Related U.S. Application Data

(60) Provisional application No. 60/087,430, filed on May 31, 1998.

(51) **Int. Cl.**[7] **G01N 33/00**; A01K 67/027; C12N 15/00; C07H 21/02; C07H 21/04

(52) **U.S. Cl.** **800/3**; 800/20; 800/21; 800/25; 536/23.1; 536/23.7

(58) **Field of Search** 800/3, 20, 21, 800/25, 18; 536/23.1, 23.5, 23.7

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,347,075		9/1994	Sorge	800/18
5,470,706		11/1995	Vijg et al.	435/6
5,510,099	*	4/1996	Short	424/9.2
5,545,808	*	8/1996	Hew et al.	800/20
5,589,155		12/1996	Sorge et al.	424/9.2
5,602,300		2/1997	Gossen et al.	800/18
5,817,290		10/1998	Vijg et al.	424/9.2
5,824,287		10/1998	Sorge et al.	424/9.2
6,114,600	*	9/2000	Ow et al.	800/278

FOREIGN PATENT DOCUMENTS

289121 B1	11/1988	(EP) .
353812 B1	2/1990	(EP) .
08205708	1/1965	(JP) .
96029093 B2	3/1996	(JP) .
8205708	8/1996	(JP) .
2618973	3/1997	(JP) .
9100567	11/1992	(NL) .
8801826	2/1998	(NL) .
WO 00/24246	5/1960	(WO) .
WO 91/15579	10/1991	(WO) .
WO 92/17605	10/1992	(WO) .
WO 93/15769	8/1993	(WO) .
WO 93/17123	9/1993	(WO) .
WO 96/37579	11/1996	(WO) .
WO 97/05484	2/1997	(WO) .
WO 99/62333	12/1999	(WO) .

OTHER PUBLICATIONS

DuBridge et al., "Recombinant Shuttle Vectors for the Study of Mutation in Mammalian Cells," *Mutagenesis*, 3(1):1–9 (1988).

Hinton et al., "Cytological Changes During Progression of Neoplasia in Selected Fish Species," *Aquat. Toxicol.*, 11:77–112 (1988).

Bearzotti et al., Gene expression following transfection of fish cells, Nov. 1992, Journal of Biotechnology, vol. 26, pp. 315–325.*

Winn et al., Transfer, Methylation and Spintaneous Mutation . . . , 1995, Marine Environmental Research, vol. 40, No. 3, pp. 247–265.*

Article: "Scientists fish for toxicity clues", *Chemistry & Industry*, pp. 131 (1999).

Bayer et al., "A Transgene Containing lacZ is Expressed in Primary Sensory Neurons in Zebrafish," *Development*, 115:421–426 (1992).

Boerrigter et al., "Plasmid–based transgenic mouse model for studying in vivo mutations", *Nature*, 377 (6550):657–659 (1995).

Boerrigter et al., "Sources of Variability in Mutant Frequency Determinations in Different Organs of lacZ Plasmid–based Transgenic Mice: Experimental Features and Statistical Analysis", *Env. Mol. Mutagenesis*, 29(3):221–229 (1997).

Boerrigter, "High Sensitivity for Color Mutants in lacZ Plasmid–Based Transgenic Mice, as Detected by Positive Selection", *Env. Mol. Mutagenesis*, 32(2):148–154 (1998).

Culp et al., "High–frequency germ–line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs", *Proc. Natl. Acad. Sci. USA*, 88(18):7953–7957 (1991).

Detrick, "Transgenic Fish Model for Mutagenicity Testing", Crisp Data Base National Institute of Health, located at website: alt1.csa.com/htbin/ids51/procskel.cgi, Abstract, 1 pg. (1994).

Dolléét al., "Evaluation of a plasmid–based transgenic mouse model for detecting in vivo mutations", *Mutagenesis*, 11(1):111–118 (1996).

Douglas et al., "Sequence spectra of spontaneous lacZ gene mutations in transgenic mouse somatic and germline tissues", *Mutagenesis*, 9(5):451–458 (1994).

Driever et al., "A genetic screen for mutations affecting embryogenesis in zebrafish", *Development*, 123:37–46 (1996).

(List continued on next page.)

Primary Examiner—Deborah J. R. Clark
Assistant Examiner—Shin-Lin Chen
(74) *Attorney, Agent, or Firm*—Mueting, Raasch & Gebhardt, P.A.

(57) **ABSTRACT**

The present invention provides transgenic fish whose somatic and germ cells contain a genomically integrated bacteriophage lambda-derived transgene construct. The transgene construct can include an excisable test nucleic acid sequence containing a heterologous mutation target nucleic acid sequence that is detectable via bioassay in a bacterial cell into which the test nucleic acid has been introduced. The frequency of mutations in the mutation target nucleic acid sequence following exposure of the transgenic fish to one or more potentially mutagenic agents can thus be evaluated.

35 Claims, 4 Drawing Sheets

OTHER PUBLICATIONS

Fosgate, Ed., "Fish may soon replace lab mice", *Forresters' Log*, pp. 6 and pp. 8 (1999).
Fosgate, "Transgenic fish may give lab mice a day off", *Georgia Magazine*, pp. 11 (1999).
Gossen et al., "Plasmid Rescue from Transgenic Mouse DNA Using LacI Repressor Protein Conjugated to Magnetic Beads", *BioTechniques*, 14(4):624–629 (1993).
Gossen et al., "Spontaneous and X–ray induced deletion mutations in LacZ plasmid–based transgenic mouse model" *Mut. Res.*, 331:89–97 (1995).
Lin et al., "lacZ Expression in Germline Transgenic Zebrafish Can Be Detected in Living Embryos," *Developmental Biology*, 161:77–83 (1994).
Riley et al., "Efficient induction of point mutations allowing recovery of specific locus mutations in zebrafish", *Proc. Natl. Acad. Sci. USA*, 92(13):5997–6001 (1995).
Rüther et al., "Easy identification of cDNA clones", *EMBO J.*, 2(10):1791–1794 (1983).
Solnica–Krezel et al., "Efficient Recovery of ENU–Induced Mutations From the Zebrafish Germline", *Genetics*, 136:1401–1420 (1994).
Stuart et al., "Replication, integration and stable germ–line transmission of foreign sequences injected into early zebrafish embryos", *Development*, 103(2):403–412 (1988).
Susman, "Gene–altered fish check new chemicals", located at website: news.chemicalonline.com/wires/19990124–421734772.htm, 2 pgs. (1999).
Tao et al., "Comparison of somatic mutation in a transgenic versus host locus", *Proc. Natl. Acad. Sci., USA*, 90(22):10681–10685 (1993).
Torassa, "Animals Hold Key to Cures", located at website: abcnews.go.com:80/sections/science/DailyNews/scorpions9990125.htm, 2 pgs. (1999).
Vijg, et al., "Ch.29: Bacteriophage Lambda and Plasmid lacZ Transgenic Mice for Studying Mutations in vivo", *Technologies for Detection of DNA Damage and Mutations*, Plenum Press, NY, Title page, publication page, table of contents and pp. 391–410 (1996).
Winn et al., "Detection of mutations in transgenic fish carrying a bacteriophage λcii transgene target", *PNAS*, 97(23):12655–12660 (2000).
"MutaPlax™ Transgenic Lambda DNA Packaging System," [online] [retrieved May 7, 1998]. Retrieved from the Internet: interscience.com/epicentre/mutplxc2.html, 1 page.
"MutaPlax cII—Select™ Packaging and Selection Kit," [online] [retrieved May 28, 1998]. Retrieved from the Internet: interscience.com/epicentre/mutplxc2.html, 1 page.
"A short description of the lambda cII system," [online]. Biology Dept., University of Victoria, Canada, [retrieved on May 8, 1998]. Retrieved from the Internet darwin.cch.uvic.ca/bigblue/cci–info.htm, 1 page.
Amanuma et al. Transgenic Zebrafish for Detectig Mutations Caused by Compounds in Aquatic Environments. Nature Biotechnology, vol. 18, pp. 62–65, Jan. 2000.*
Murray, J.D. et al. Transgenic Animals in Agriculture. CABI Publishing. New York, 1999.*
Houdebine, L.M. et al. Transgenesis in Fish. Experientia 47(9): 891–897, Sep. 1991.*
Liu, Z. et al. Development of Expression Vectors for Transgenic Fish. Bio/Technology 8:1268–1272, Dec. 1990.*
Lubzens, E. et al. Cryopreservation as a tool in aquaculture. Israeli Journal of Aquaculture 44(4):137, 1992.*
Bailey et al., "Initiation, Promotion, and Inhibition of Carcinogenesis in Rainbow Trout," *Environ. Health Perspect.* 71:147–153 (1987).
Barnes, "Fish Embryonal Stem Cells for Development of Transgenics", Abstract, Grant No. 5 R01 ES06011–06, 1 pg. (no later than Mar. 1998).
Black, "Aquatic Animal Neoplasia as an Indicator for Carcinogenesis Hazards to Man," *Hazard Assessment of Chemicals: Current Developments* vol. 3, Saxena, ed., Academic Press, New York. 181–232 (1984).
Burkhart et al., "Non–Mammalian and Environmental Sentinels in Human Health: Back to the Future?" *Human and Ecological Risk Assessment* vol. 3, CRC Press LLC, 309–328 (1997).
Burkhart et al., "Spontaneous and Induced Mutagenesis in Transgenic Animals Containing ϕX174," *Environ. Mol. Mut.* 21:8–9 Abstract Only (1993).
Burkhart et al., "ENU–induced Mutagenesis at a Single A : T Base Pair in Transgenic Mice Containing ϕX174," *Mutation Res.* 292:69–81 (1993).
Burkhart, "Comparative Mutagenesis in Aquatic Models, Rodents, and Cells in Culture," Abstract, Grant Z01 ES21187–02, 1 pg. (no later than Mar. 1998).
Chen, "Transgenic Fish a Model for Environmental Toxicology," Abstract, Grant No. 5 R01 RR11680–03, 1 pg. (no later than Mar. 1998).
Driever et al., "Zebrafish: Genetic Tools for Studying Vertebrate Development," *Trends Genet.* 10(5):152–159 (1994).
Dycaico et al., "The Use of Shuttle Vectors for Mutation Analysis in Transgenic Mice and Rats," *Mutation Res.* 307:461–478 (1994).
Gallagher et al., "A Comparative Approach to 7,12–Demethylbenz[A]Anthracene Effects: Metabolism and Mutagenesis in Mice and Fish," *Environ. Mol. Mut.* 29:15–16, Abstract Only (1997).
Goldsworthy et al., "Transgenic Animals in Toxicology," *Fund Appl. Toxicol.* 22:8–19 (1994).
Gossen et al., "Efficient Rescue of Integrated Shuttle Vectors from Transgenic Mice: A Model for Studying Mutations In Vivo," *Proc. Natl. Acad. Sci.* 86:7971–7975 (1989).
Grunwald, "Embryo Stem Cell Cultures and Gene Transfer in Zebrafish," Abstract, Grant No. 5 R01 RR10253–04, 1 pg. (no later than Mar. 1998).
Gu et al., "Development of a λ–based Complementation Assay for the Preliminary Localization of lacI mutants from the Big Blue ™ Mouse: Implications for a DNA–Sequencing Strategy," *Mutation Res.* 307:533–540 (1994).
Guyomard et al., "Integration and Germ Line Transmission of Foreign Genes Microinjected into Fertilized Trout Eggs," *Biochemie*, 71:857–863 (1989).
Hawkins et al., "Carcinogenicity Tests Using Aquarium Fish," *Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment*, Chapter 14 in G.M. Rand, ed., Taylor and Francis, 421–446 (1995).
Hendricks, "Chemical Carcinogenesis in Fish," *Aquatic Toxicology*, Weber, ed., Raven Press, New York., 149–211 (1982).
Inoue et al., "Stage–dependent Expression of the Chicken δ–crystallin Gene in Transgenic Fish Embryos," *Cell Differ. Dev.* 27(1):57–68 (1989).
Inoue et al., "Electroporation as a New Technique for Producing Transgenic Fish," *Cell Differ. Dev.* 29(2):123–128 (1990).

Ishikawa et al., "Usefulness of the Medaka, *Oryzias latipes*, as a Test Animal: DNA Repair Processes in Medaka Exposed to Carcinogens," *Natl. Cancer Inst. Monograph* 65:35–43 (1984).

Ishikawa et al., "Importance of Hepatic Neoplasms in Lower Vertebrate Animals as a Tool in Cancer Research," *J. Toxicol. Environ. Health* 5:537–550 (1997).

Jakubczak et al., "Analysis of Genetic Instability During Mammary Tumor Progression Using a Novel Selection–based Assay for In Vivo Mutations in Bacteriophage Lambda Transgene Target," *Proc. Natl. Acad. Sci. USA* 93:9073–9078 (1996).

Kaiser, "Fishing for Toxic Chemicals," *Science* 283:775–777 (1999).

Kohler et al.,"Spectra of Spontaneous and Mutagen–Induced Mutations in the lacI Gene in Transgenic Mice," *Proc. Natl. Acad. Sci. USA* 88:7958–7962 (1991).

Lebkowski et al., "The lacI Shuttle: Rapid Analysis of the Mutagenic Specificity of Ultraviolet Light in Human Cells," *Proc. Natl. Acad. Sci.* 82:8606–8610 (1985).

Lewis et al., "The Nature of Spontaneous and Induced Electrophoretically Detected Mutations in the Mouse," *Prog. Clin. Biol. Res.* 209B:359–365 (1986).

Lu et al., "Pantropic Retroviral Vector Integration, Expression, and Germline Transmission in Medaka (*Oryzias latipes*)," *Mol. Mar. Biol. Biotechnol.* 6(4):289–295 (1997).

Lu et al., "Integration, Expression, and Germ–line Transmission of Foreign Growth Hormone Genes in Medaka (*Oryzias latipes*)," *Mol. Marine Biol. and Biotechnol.* 1(4/5):366–375 (1992).

Masahito et al., "Fish Tumors and Their Importance in Cancer Research," *Jpn. J. Cancer Res.* 79:545–555 (1988).

Malling et al., "Use of φX174 as a Shuttle Vector for the Study of In Vivo Mammalian Mutagenesis," *Mutation Res.* 212:11–21 (1989).

Metcalfe, "Tests for Predicting Carcinogenicity in Fish," *CRC Rev. Aquat. Sci.* 1:111–129 (1989).

Mirsalis et al., "Induction of Hepatic Mutations in lacI Transgenic Mice," *Mutagenesis* 8:265–271 (1993).

Mirsalis et al., "Transgenic Animal Models for Detection of In Vivo Mutations," *Ann. Rev. Pharmacol. Toxicol.* 35:145–164 (1995).

Müller et al., "Introducing Foreign Genes into Fish Eggs with Electroporated Sperm as a Carrier," *Mol. Mar. Biol. Biotechnol.* 1 (4–5):276–281 (1992).

Müller et al., "Efficient Transient Expression System Based on Square Pulse Electroporation and In Vivo Luciferase Assay of Fertilized Fish Eggs," *FEBS Lett.* 324(1):27–32 (1993).

Murakami et al., "Micromachined Electroporation System for Transgenic Fish," *J. Biotechnol.* 34(1):35–42 (1994).

Nebert, "Transgenic Zebrafish–Sentinel for Aquatic Pollution", Abstract, Grant No. 5 R01 ES07058–02, 1 pg. (no later than Mar. 1998).

Ozato et al., "Production of Transgenic Fish: Introduction and Expression of Chicken δ–crystallin Gene in Medaka Embryos," *Cell Differ.* 19:237–244 (1986).

Postlethwait et al., "Zebrafish Genomics: From Mutants to Genes," *Trends Genet.* 13(5):183–190 (1997).

Powers et al., "Electroporation: A Method for Transferring Genes into the Gametes of Zebrafish (*Brachydanio rerio*), Channel Catfish (*Ictalurus punctatus*), and Common Carp (*Cyprinus carpio*)," *Mol. Mar. Biol. Biotechnol.* 1(4–5):301–308 (1992).

Powers, "Fish as Model Systems," *Science* 246:352–358 (1989).

Provost et al., "Transgenic Systems for In Vivo Mutation Analysis," *Mutation Res.* 288:133–149 (1993).

Rogers et al., "Intralaboratory Optimization and Standardization of Mutant Screening Conditions Used for a Lambda/lacI Transgenic Mouse Mutagenesis Assay(I)," *Mutation Res.* 327:57–66 (1995).

Rokkones et al., "Microinjection and Expression of a Mouse Metallothionein Human Growth Hormone Fusion Gene in Fertilized Salmonid Eggs," *J. Comp. Physiol. B.*, 158:751–758 (1989).

Russell et al., "The Mouse Specific–Locus Test with Agents Other than Radiations, Interpretation of Data and Recommendations for Future Work," *Mutation Res.* 86:329–354 (1981).

Sambrook et al., "Preparation of Reagents and Buffers Used in Molecular Cloning," *Molecular Cloning* 2nd Ed., Cold Spring Harbor Press, p.B.13 (1989).

Schwarz et al., Nucleotide Sequence of cro, cII and Part of the o gene in phage λ DNA *Nature* 272:410–414 (1978).

Shimada et al., "Some Characteristics of Radiation– or Enu–induced Mutations at the b Locus of the Medaka," *Phys. Gen.* 7(6):1053 (1990).

Shimada et al., "Further Characterization of Radiation– or Enu–induced Mutations at the b Locus of the Medaka," *Genetics* 8(6):1127 (1991).

Singer, "All Oxygens in Nucleic Acids React with Carcinogenic Ethylating Agents," *Nature* 264:333–339 (1976).

Singer et al., "Oxygens in DNA are Main Targets for Ethylnitrosourea in Normal and *Xeroderma Pigmentosum* Fibroblasts and Fetal Rat Brain Cells," *Nature* 276:85–88 (1978).

Skopek et al., "Relative Sensitivity of the Endogenous hprt Gene and lacI Transgene in ENU–Treated Big Blue™ B6C3F1 Mice," *Environ. Mol. Mut.* 26(1):9–15 (1995).

Streisinger et al., "Production of Clones of Homozygous Diploid Zebra Fish (*Brachydanio rerio*), " *Nature* 291:293–296 (1981).

Symonds et al., "Development of a Mass Gene Transfer Method in Chinook Salmon: Optimization of Gene Transfer by Electroplated Sperm," *Mol. Mar. Biol. Biotechnol.* 3(2):104–111 (1994).

Szelei et al., "Entrapment of High–Molecular–Mass DNA Molecules in Liposomes for the Genetic Transformation of Animal Cells," *Biochem. J.* 259:549–553 (1989).

Tamiya et al., "Spatial Imaging of Luciferase Gene Expression in Transgenic Fish," *Nucleic Acids Res.* 18:1072 (1990).

Tennant et al., "Prediction of Chemical Carcinogenicity in Rodent from In Vitro Genetic Toxicity Assays," *Science* 236:933–941 (1987).

Tsai et al., "Initiation of the Transgenic lacZ Gene Expression in Medaka (*Oryzias latipes*) Embryos," *Mol. Mar. Biotechnol.* 4(1):1–9 (1995).

Valcovic et al., "An Approach to Measuring Germinal Mutations in the Mouse," *Environ. Health Perspect.* 6:201–205 (1973).

Van Beneden et al., "Oncogenes in Hematopoietic and Hepatic Fish Neoplasms," *Cancer Res.* 50:5671s–5674s (1990).

Vogelstein et al., "Genetic Alterations During Colorectal–Tumor Development," *N. Eng. J. Med.* 319(9):525–532 (1988).

Walker et al., "A Small Fish Model for Assessing Cancer Risk at Low Carcinogen Concentrations," *Toxicologist* 12, Abstract No. 302 (1992).

Winn et al., "Transfer, Methylation and Spontaneous Mutation Frequency of ϕX174am3cs70 sequences in Medaka (*Oryzias latipes*) and Mummichog (*Fundulus heteroclitus*): Implications for Gene Transfer and Environmental Mutagenesis in Aquatic Species," *Marine Environ. Res.* 40(3):247–265 (1995).

Winn, "Transgenic Fish Model for Mutation Detection", Abstract, Grant No. 1 R24 RR11733–01A1, 1 pg., (no later than Mar. 1998).

Yamamoto, "Medaka (Killifish): Biology and Strains," Keigaku Publishing Co., Toyko, Japan, Title Page and Table of Contents (1975).

Zelenin et al., "The Delivery of Foreign Genes into Fertilized Fish Eggs Using High–Velocity Microprojectiles," *FEBS Lett.* 287(1–2):118–120 (1991).

International Agency for Research on Cancer (IARC), IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, 7, IARC, Lyon., 253–260 (1974).

"λ Select–cII™ Mutation Detection System for the Big Blue® Rodents," Instruction Manual, Stratagene, Catalog #720120, Revision #028001, p. 1–21 (1998).

"cII Primers Product" Information Sheets, Epicentre Technologies, Catalog #P67PL1, and #P67PL2, 1 pg. (1996).

Big Blue™ Transgenic Mouse Mutagenesis Assay System, Instruction Manual Title Page and Table of Contents, 2 pgs. (Aug. 1992).

http://www.stratagene.com/vol10_3/figures/p100–101.htm, "Big Blue™ γLIZ Shuttle Vector", 1 pg. (May 1998).

http://www.stratagene.com/vol10_3/figures/p100–101–fl.htm, "A Positive Selection Assay for Mutation Analysis in Big Blue™ Animals", 2 pgs. (May 1998).

http://www.stratagene.com/tech_ref/big_blue/faq.htm, "Technical Information—Stratagene Online", 3 pgs. (Mar. 1998).

http://darwin.ceh.uvic.ca/bigblue/bbinfo.htm, "A short description of the Big Blue system", 1 pg. (May 1998).

http://darwin.ceh.uvic.ca/bigblue/cci–info.htm, "A short description of the lambda cII system", 1 pg. (May 1998).

http://www.interscience.com/epicentre/mutplxc2.html, "MutaPlax™ Transgenic Lambda DNA Packaging System", 1 pg. (May 1998).

http://www.interscience.com/epicentre/mutplxc2.html, "MutaPlax cII–Select™ Packaging and Selection Kit", 1 pg. (May 1998).

* cited by examiner

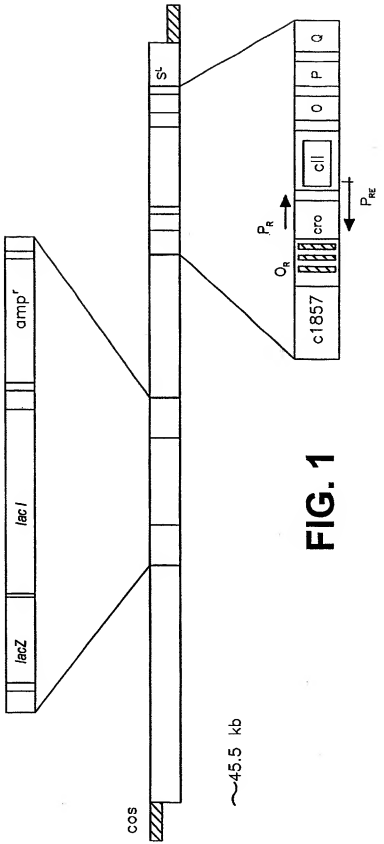


FIG. 1

A

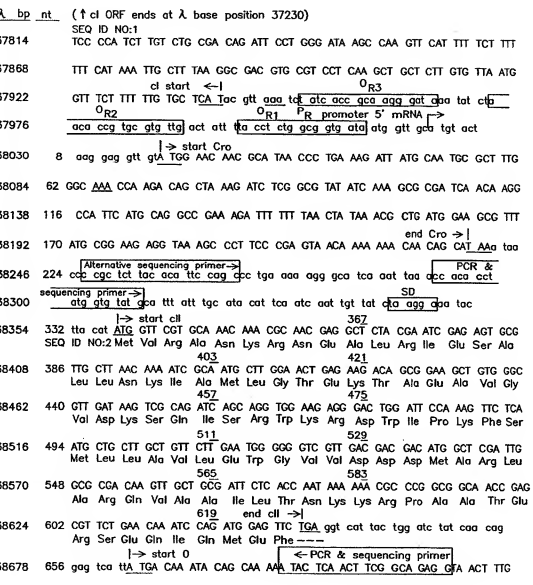

λ bp nt (↑ cI ORF ends at λ base position 37230)
SEQ ID NO:1
37814 TCC CCA TCT TGT CTG CGA CAG ATT CCT GGG ATA AGC CAA GTT CAT TTT TCT TTT
37868 TTT CAT AAA TTG CTT TAA GGC GAC GTG CGT CCT CAA GCT GCT CTT GTG TTA ATG
cI start
37922 GTT TCT TTT TTG TGC TCA Tac gtt aaa tat atc acc gca agg gat gaa tat ctg
37976 aca ccg tgc gtg ttg act att tta cct ctg gcg gtg ata atg gtt gca tgt act
start Cro
38030 8 aag gag gtt gtA TGG AAC AAC GCA TAA CCC TGA AAG ATT ATG CAA TGC GCT TTG
38084 62 GGC AAA CCA AGA CAG CTA AAG ATC TCG GCG TAT ATC AAA GCG CGA TCA ACA AGG
38138 116 CCA TTC ATG CAG GCC GAA AGA TTT TTT TAA CTA TAA ACG CTG ATG GAA GCG TTT
end Cro
38192 170 ATG CGG AAG AGG TAA AGC CCT TCC CGA GTA ACA AAA AAA CAA CAG CAT AAa taa
38246 224 ccc cgc tct tac aca ttc cag gcc tga aaa agg gca tca aat taa acc aca cct
38300 atg gtg tat gca ttt att tgc ata cat tca atc aat tgt tat cta agg gaa tac
start cII
38354 332 tta cat ATG GTT CGT GCA AAC AAA CGC AAC GAG GCT CTA CGA ATC GAG AGT GCG
SEQ ID NO:2 Met Val Arg Ala Asn Lys Arg Asn Glu Ala Leu Arg Ile Glu Ser Ala
38408 386 TTG CTT AAC AAA ATC GCA ATG CTT GGA ACT GAG AAG ACA GCG GAA GCT GTG GGC
Leu Leu Asn Lys Ile Ala Met Leu Gly Thr Glu Lys Thr Ala Glu Ala Val Gly
38462 440 GTT GAT AAG TCG CAG ATC AGC AGG TGC AAG AGG GAC TGG ATT CCA AAG TTC TCA
Val Asp Lys Ser Gln Ile Ser Arg Trp Lys Arg Asp Trp Ile Pro Lys Phe Ser
38516 494 ATG CTG CTT GCT GTT CTT GAA TGG GGG CTC GTT GAC GAC GAC ATG GCT CGA TTG
Met Leu Leu Ala Val Leu Glu Trp Gly Val Val Asp Asp Asp Met Ala Arg Leu
38570 548 GCG CGA CAA GTT GCT GCG ATT CTC ACC AAT AAA AAA CGC CCG GCG GCA ACC GAG
Ala Arg Gln Val Ala Ala Ile Leu Thr Asn Lys Lys Arg Pro Ala Ala Thr Glu
end cII
38624 602 CGT TCT GAA CAA ATC CAG ATG GAG TTC TGA ggt cat tac tgg atc tat caa cag
Arg Ser Glu Gln Ile Gln Met Glu Phe
start 0
38678 656 gag tca ttA TGA CAA ATA CAG CAA AAA TAC TCA ACT TCG GCA GAG GTA ACT TTG
λ promoter 5' mRNA
Alternative sequencing primer
PCR &
sequencing primer
SD
PCR & sequencing primer

FIG. 2

B

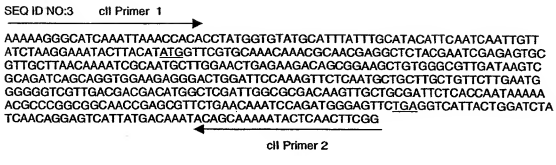


Fig. 2 (continued)

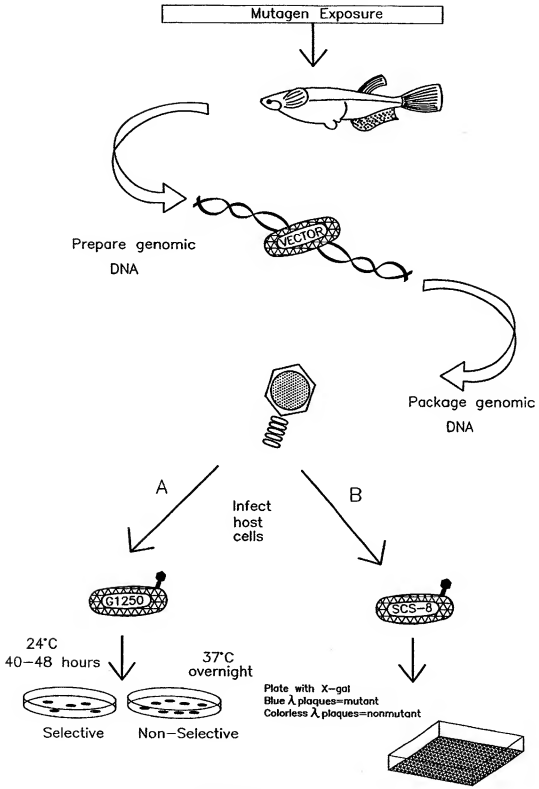

Mutagen Exposure
Prepare genomic DNA
VECTOR
Package genomic DNA
A
Infect host cells
B
G1250
SCS-8
24°C 40-48 hours
37°C overnight
Selective
Non-Selective
Plate with X-gal
Blue λ plaques=mutant
Colorless λ plaques=nonmutant

FIG. 3

BACTERIOPHAGE-BASED TRANSGENIC FISH FOR MUTATION DETECTION

This application claims the benefit of U.S. Provisional Application No. 60,087,430, filed May 31, 1998.

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with government support under a grant from the National Institutes of Health, Grant No. RR11733-01. The U.S. Government has certain rights in this invention.

FIELD OF THE INVENTION

This invention relates to a transgenic fisb carrying a bacteriophage-derived transgene construct, and in particular relates to a transgenic fish for use in evaluating the effect of a potential mutagenic agent or event. The transgenic fish is exposed to the mutagenic agent or event, and mutagenesis is detected by assaying for a mutation target nucleic acid sequence present as a genomically integrated transgene in the transgenic fish.

BACKGROUND OF THE INVENTION

Thousands of chemicals are currently in commercial use in the USA, some of which pose significant bealth risk to humans. Among these toxicants are mutagens to which exposure is likely to cause genetic changes that lead to somatic or inherited diseases. In particular, cancer has been shown to result from a series of mutations in specific oncogenes and tumor suppressor genes (B. Vogelstein et al., *N. Engl. J. Med.* 319: 525–532 (1988)). Despite the recognition of the role of chemically-induced mutation as an important event leading to disease, few methods are available for the assessment of genetic hazard, or focus on the study of gene mutations as they occur at the DNA level in vivo. As a result, there is an immediate need to develop sensitive and biologically relevant methods that can be applied to the study of the mechanisms of mutagenesis and hazard assessment.

Until recently, progress in the analysis of gene mutations directly at the DNA level was limited by the standard molecular techniques and the available endogenous genes. During past years, the most relevant assays for induction of transmissible mutations have been based on the appearance of visible or biochemical mutations among the offspring of exposed mice (L. B. Russell et al., *Mutation Res.* 86: 329–354 (1981); L. R. Valcovic et al., *Environ. Health Perspect.* 6:201–205 (1973); S. E. Lewis et al., *Prog. Clin. Biol. Res.* 209B. 359–365 1986)). These tests cannot be practically applied to large numbers of compounds because they require extensive resources and very large numbers of animals. The tests also fail to provide information regarding somatic mutagenesis or clustering of mutations, which may be important in the understanding of the development of various diseases.

In order to circumvent some of the problems inherent in rodent assays, short-term mutagenicity tests were developed, based on the assumption that many of the chemicals toxic to rodents would also be genotoxic to bacteria. However, an analysis by the National Toxicology Program (R. W. Tennant et al., *Science* 236:933–941 1987)) revealed significant differences in results between rodent and bacterial tests. This failure of predictive correlation may be related to: 1) a lack of understanding of the roles mutation plays in cell transformation, and 2) differences between animals and bacterial cells in terms of exposure, biological milieu, metabolism, replication and repair. While comparisons between animals and animal cells in culture provide appropriate genomic similarity, there are few known biological markers for mutation of cells in culture. The biological markers that have been identified are restricted to specific cell types and therefore are of limited use for in vivo comparisons.

There thus remains a need to combine the simplicity of short-term in vitro assays with in vivo studies. Ultimately, reliable and realistic hazard assessment and informative mechanistic studies of mutagenesis require the development of practical methods for evaluating somatic and genetic events in whole animals exposed to environmental agents. New approaches that use recombinant DNA and gene transfer techniques to develop transgenic animal models offer significant promise for in vivo studies of mutagenesis, cancer, birth defects and otber diseases (T. L. Goldsworthy et al., *Fund. Appl. Toxicol.* 22:8–19 (1994)). To be effective, the transgenic approach as applied to mutagenesis should include the following components: 1) unique genes with known sequences; 2) a capacity to observe changes at the single copy level; 3) an easily attainable sample population of sufficient size to allow measurement of low frequency events; and 4) the ability to determine the exact nature of the mutation, independent of tbe host phenotype.

Transgenic animal models have been developed. Typically, transgenic animals are produced by the transfer of novel DNA sequences into tbe animal's genome followed by transmission of the sequence to subsequent generations. The use of transgenic rodents that carry genes specifically designed for the quantitation of spontaneous and induced mutations is a major advancement in rapidly analyzing tissue-specific mutations in a whole organism following mutagenic agent exposure (J. C. Mirsalis et al., *Ann. Rev. Pharmacol. Toxicol.* 35:145–164 1995)).

Mutagenesis assay systems that utilize transgenic animals typically rely on bacteriopbage or plasmid shuttle vectors to carry the mutation target. The basic principle in this approach is that a recombinant gene carrying a mutation target is introduced into the genome of a host animal using the shuttle vector. Following exposure to a mutagen, the target gene is recovered from tbe transgenic animal and serves as an indicator of mutagenesis (reviewed by R. B. Dubridge et al., *Mutagenesis* 3(1):1–9 (1988)). Two forms of bacteriophage sbuttle vectors are most commonly in use. One is known as the ψX174 integrated shuttle vector. This vector is recovered from the transgenic host, transfected into a suitable *E. coli* host, and mutations at specific locations in the phage sequence are identified by suppressor-mediated selection on permissive and non-permissive *E. coli* (H. V. Malling et al., *Mutation Res.* 212:1–21 (1989); R. N. Winn et al., *Marine Environ. Res.* 40(3):247–265 (1995)).

Another useful mutagenesis detection system is based on a lambda (λ) phage-based recombinant vector which combines cos site packaging for recovery of the phage sequence from the host DNA with tbe use of the lacI or lacZ target gene for mutation detection (J. S. Lebkowski et al., *Proc. Natl. Acad. Sci.* 82:8606–8610 (1985); J. A. Gossen et al., *Proc. Natl. Acad. Sci.* 86:7971–7975 (1989)).

Mutation-induced inactivation of the lac genes is detected after recovery of the shuttle sequence from the transgenic host, typically via complementation assay in *E. coli*. See U.S. Pat. No. 5,589,155 (Sorge et al., Dec. 31, 1996); U.S. Pat. No. 5,347,075 (Sorge, Sep. 13, 1994); and U.S. Pat. No. 5,510,099 (Short et al., Apr. 23, 1996), the texts of which are

incorporated by reference, in their entireties, as if fully set forth herein. A mutation detection system based on the lacI gene as the mutation target, known by the tradename BIG BLUE, is commercially available from Stratagene Inc. (La Jolla, Calif.). The vector used in BIG BLUE mutagenesis detection system is known as λLIZ, and the genetic map of this vector is shown in FIG. 1.

The λLIZ vector contains an additional mutagenesis target in the form of the cII region (see FIG. 1). Mutations in the cII gene and at certain related locations in the cII region can be detected by evaluating whether an *E. coli* host that has been infected with the shuttle sequence recovered from the transgenic host can multiply through the lytic or the lysogenic cycle (J. L. Jakubczak et al., *Proc. Natl. Acad. Sci. U.S.A.*, 93:9073–9078 (1996)). The commitment to either lysis or lysogeny made by lambda phage upon infection of an *E. coli* cell is regulated by a group of proteins, one of which is the product of the cII gene. Mutagenesis detection packaging and selection systems based upon the cII region as the mutation target sequence are commercially available from Stratagene Inc., La Jolla, Calif. (available under tradename λ SELECT-cII) and Epicentre Technologies, Madison, Wis. (available under the tradename MutaPlax cII-Select Packaging and Selection Kit).

To date, the lambda (λ) phage-based recombinant vectors disclosed in U.S. Pat. Nos. 5,589,155, 5,347,075, and 5,510, 099; European Patent No. 0289121; and Japanese Patent No. 2618973, each of which is incorporated herein in its entirety, have been used successfully for detection of mutagenic events only in selected mammalian cell lines, mice and rats. Alternative animal models are thus much needed. Fish as transgenic hosts are especially desirable because they are environmentally relevant models for health risk assessment of aquatic and marine systems. There is also increasing appreciation of their suitability for biomedical applications. The fish is an alternative, nonmammalian animal model that can be used to refine, reduce or replace traditional animal models used in research and testing.

SUMMARY OF THE INVENTION

The present invention provides a transgenic fish containing a genomically integrated bacteriophage lambda-derived transgene construct. The invention further includes a transgenic fish gamete, including a transgenic fish egg or sperm cell, a transgenic fish embryo, or other transgenic fish cell or cluster of cells, whether haploid, diploid, triploid, or other zygosity, which contain a genomically integrated bacteriophage lambda-derived transgene construct. A transgene construct that is "bacteriophage lambda-derived" is a construct that is based on the bacteriophage lambda. Preferably, the bacteriophage lambda-derived transgene construct is genomically integrated into the fish's somatic and germ cells such that it is stable and inheritable. Progeny of a transgenic fish containing a genomically integrated bacteriophage lambda-derived transgene construct, and transgenic fish derived from a transgenic fish egg, sperm cell, embryo, or other cell containing a genomically integrated bacteriophage lambda-derived transgene construct, are also included in the invention. A fish is "derived from" a transgenic fish egg, sperm cell, embryo or other cell if the transgenic fish egg, sperm cell, embryo or other cell contributes DNA to the fish's genomic DNA. For example, a transgenic embryo of the invention can develop into a transgenic fish of the invention; a transgenic egg of the invention can be fertilized to create a transgenic embryo of the invention that develops into a transgenic fish of the invention; a transgenic sperm cell of the invention can be used to fertilize an egg to create a transgenic embryo of the invention that develops into a transgenic fish of the invention; and a transgenic cell of the invention can be used to clone a transgenic fish of the invention. In some preferred embodiments of the invention, the transgenic fish is sterile.

The transgenic fish of the invention is preferably a teleost (bony) fish, but also includes a cartilagenous fish. Conveniently, the transgenic fish can be selected from among the well-known group of laboratory model fish which include medaka, zebrafish, mummichog, killifish, channel catfish, common carp and trout. In a particularly preferred embodiment, the transgenic fish is a medaka.

Further, the present invention includes a cell line derived from a transgenic fish embryo or other transgenic fish cell of the invention, which contains a genomically integrated bacteriophage lambda-derived transgene construct.

The bacteriophage lambda-derived transgene construct preferably comprises an excisable test nucleic acid sequence that contains at least one copy of an assayable mutation target nucleic acid sequence. The assayable mutation target nucleic acid sequence is heterologous with respect to the fish genome. Two or more different assayable mutation target nucleic acid sequences can optionally be present in the bacteriophage lambda-derived transgene construct. In a particularly preferred embodiment, a mutation in the assayable mutation target nucleic acid sequence is detectable via bioassay in a bacterial cell, such as an *E. coli* cell, into which the assayable mutation target nucleic acid sequence has been introduced. In this regard, a transgenic fish of the invention that has a triploid genome is especially preferred because the larger amount of DNA in triploid genomes increases the efficiency of DNA recovery. An increase in the amount of DNA recovered has many advantages. For example, it allows for more efficient detection of the mutation target nucleic acid. Moreover, fish having a triploid genome are typically sterile, which may be desirable for certain applications or studies.

The assayable mutation target nucleic acid sequence preferably contains at least one nucleic acid sequence selected from the group consisting of the lacI gene, the lacZ gene, the lac promoter sequence, the cII gene, the cII mRNA ribosome binding site, and the cII protein-activated P_{RE} promoter.

The invention further includes a genomically identical population of transgenic fish, each of whose somatic and germ cells contain a genomically integrated bacteriophage lambda-derived transgene construct. The genomically identical population is a unisex population and can be male or female. Preferably, the genomically integrated bacteriophage lambda-derived transgene construct present in the genomically identical population contains an excisable test nucleic acid sequence comprising at least one copy of one or more assayable mutation target nucleic acid sequences. Preferred embodiments of the genomically identical transgenic fish population are essentially as described for the transgenic fish of the invention. In an alternative embodiment, the invention includes a population of transgenic fish, i.e., an in-bred line, the members of which are not necessarily genomically identical but are homozygous with respect to the bacteriophage lambda-derived transgene construct.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic diagram of the BIG BLUE λLIZ shuttle vector (~45.5 kb) (Stratagene, Inc., La Jolla, Calif.); the expanded regions show (A) the area important for a

plaque-color-screening assay to detect mutations, which contains lacI, and (B) the area important for a selection assay based upon mutation in the cII region.

FIGS. 2A and 2B depict (A) the nucleotide sequence (SEQ ID NO:1) of the lambda cII gene and the surrounding regions in the BIG BLUE λLIZ Shuttle Vector, as shown at page 15 in the Instruction Manual for the λ Select-cII Mutation Detection System for Big Bhe Rodents (Catalog #7210210, Revision #028001, Stratagene, La Jolla, Calif.), and the amino acid sequence (SEQ ID NO:2) of the cII protein; and (B) the nucleotide sequence of the lambda cII gene (SEQ ID NO:3) and location of primer binding sites, taken from cII Primers Product Information Sheet (Cat. Nos. P67PL1, P67PL2, Epicentre Technologies, Madison, Wis.).

FIG. 3 is schematic diagram that illustrates selection assays based on the mutations in the (A) cII region and the (B) lac region of the BIG BLUE λLIZ shuttle vector shown in FIG. 1.

DETAILED DESCRIPTION OF THE INVENTION

Host Organism

There are many advantages to the use of fish to detect mutagenic agents or events. Fish are easily handled, manipulated, and observed without compromising natural development, and present opportunities for studies on multiple exposure routes via aqueous media. They exhibit excellent agent-specific responses to a variety of toxicants. Teleost fish, also known as the modern boney fishes, constitute the largest and most diverse division of vertebrates, with over 20,000 known species. Their diversity and phylogenetic positions make them ideal for comparative toxicological studies, which may allow more insight into basic mechanisms than would studies limited to mammalian models alone (D. A. Powers, *Science* 246:352–358 (1989)). Certain teleost fish, commonly referred to as laboratory aquarium fish or laboratory model species, have been extensively studied in research settings and are thus especially well-suited as transgenic hosts. Laboratory model species include, but are not limited to, medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), mummichog (*Fundulus heteroclitus*), killifish (Fundulus spp.), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*) and trout. Cartilagenous fish such as sharks and rays, also known as chondrichthyes fish, are also suitable transgenic host organisms.

In addition to playing an important role in comparative mutagenesis studies, it is anticipated that the transgenic fish models may ultimately facilitate a broad range of exposure regimens such as long-term low-dose chronic exposures, controlled field-based in situ exposures or large-scale mesocosms, that were previously difficult or impossible to perform. Fish can be conveniently used for zygote-to-adult exposure studies, and offer flexibility in study designs related to numbers of exposure groups and exposure schedules.

Fish eggs are large, abundant, and often translucent, and can be fertilized in vitro. The resulting embryos are easily maintained and develop externally, obviating the need for reintroduction of the embryo into a receptive female. In addition, laboratory analyses can generally be performed more rapidly and at lower cost compared to rodent assays.

Heterologous genes have been introduced into fish beginning in 1985. Among these heterologous genes that have been introduced into fish include genes that code for growth hormones (human, rat, rainbow trout), chicken delta-crystalline protein, *E. coli* β-galactosidase, *E. coli* neomycin-resistance, and anti-freeze protein. However, numerous problems have been encountered in producing transgenic fish having stable, inheritable genomically integrated transgenes. For example, mosaicism is a common problem in the creation of transgenic fish. Mosaic organisms do not contain the transgene in every somatic and germ cell, and thus may not be capable of producing transgenic offspring. Mosiacism arises from fact that microinjection of heterologous DNA into a fish embryo often delivers the heterologous DNA to the cytoplasm rather than the cell nucleus.

Despite the evolutionary distance between fish and humans, there is increasing evidence for correlation between environmentally-induced fish and human diseases. Fish can be exposed to different concentrations of known or suspected toxicants and can provide fundamental information related to substance toxicity and carcinogenicity/mutagenicity. The use of fish in carcinogenesis research, in particular, has received considerable attention related to the potential of fish for identifying and predicting human health effects (W. F. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor and Francis. 421–446 (1995); J. D. Hendricks, In L. J. Weber, ed., Aquatic Toxicology, Raven Press, New York. 149–211 (1982); J. J. Black, In J. Saxena, ed., Hazard Assessment of Chemicals: Current Developments, Vol. 3. Academic Press, New York. 181–232 (1984); C. D. Metcalfe, *CRC Rev. Aquat. Sci.* 1:111–129 (1989)). The fact that many fish species appear to be sensitive to the carcinogenic effects of certain chemicals while having low spontaneous rates of neoplasia supports the use of fish in various assays as alternatives or supplements to rodent chronic bioassays (G. D. Bailey et al., *Environ. Health Perspect.* 71:147–153 (1987); T. Ishikawa et al., *J. Toxicol. Environ. Health* 5:537–550 (1977); P. Masahito et al., *Jpn. J Cancer Res.* 79:545–555 (1988)).

The genetics, developmental biology and embryology of medaka (*Oryzias latipes*) are well-documented, and specific developmental stages have been extensively characterized (T. O. Yamamoto, Medaka (killifish): Biology and Strains. Keigaku Publishing Co., Tokyo, Japan. (1975)). Medaka is typically used to study aspects of various diseases in which large numbers of experimental organisms are required, such as in low-dose risk assessment, as well as to examine factors that only slightly increase hazard exposure risk (W. W. Walker, W. E. Hawkins, R. M. Overstreet, and M. A. Friedman, "A small fish model for assessing cancer risk at low carcinogen concentrations," *Toxicologist* 302 (1992)). The use of medaka in biomedical research, especially as a carcinogenesis model related to the potential for identifying and predicting human effects from toxicant exposure, has received considerable attention in recent years (W. E. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor and Francis. 421–446 (1995); J. D. Hendricks, In L. J. Weber, ed., Aquatic Toxicology, Raven Press, New York. 149–211 (1982); J. J. Black, In J. Saxena, ed., Hazard Assessment of Chemicals: Current Developments, Vol. 3. Academic Press, New York. 181–232 (1984); and C. D. Metcalfe, *CRC Rev. Aquat. Sci.* 1:111–129 (1989)). The sensitivity of medaka to many carcinogens, the availability of specimens, and the degree of control that can be maintained over extraneous factors all contribute to this small fish being one of the most widely used species for studies in comparative toxicology (W. E. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk

Assessment, Taylor and Francis. 421–446 (1995)), the biology of hepatic neoplasia (D. E. Hinton et al., *Aquat. Toxicol.* 11:77–112 (1988)), oncogene activation (R. J. Van Beneden et al., *Cancer Res.* 50:5671s–5674s (1990)), DNA repair (T. Ishikawa et al., *Natl. Cancer Inst. Monograph* 65:35–43 (1984)), and mutagenesis (R. N. Winn et al., *Marine Environ. Res.* 40(3):247–265 (1995)).

Medaka offer numerous advantages for transgenic development such as small size (about 2.5 cm), relatively short generation time (1–2 months), and prolific capacity to reproduce (more than 3,000 eggs/female in a single breeding season). Spawning can be induced year-round by maintaining breeding stocks at 25–28° C. and eggs usually hatch in 10 days at 25° C. Eggs are translucent, which greatly facilitates the positioning of fine glass needles for DNA microinjection. Medaka was the first transgenic fish species produced to demonstrate successful foreign gene expression (K. Ozato et al., *Cell Differ.* 19:237–244 (1986)). Subsequently, numerous transgenic medaka have been produced that carry a variety of transgenes (e.g. K. Inoue et al., *Cell Differ. Dev.* 27(1):57–68 (1989); E. Tamiya et al., *Nucleic Acids Res.* 18:1072 (1990); K. Inoue et al., *Cell Differ. Dev.* 29(2):123–128 (1990); J. Lu et al., *Mol. Marine Biol. and Biotechnol.* 1(4/5):366–375 (1992); H. J. Tsu et al., *Mol. Mar. Biol. Biotechnol.* 4(1):1–9 (1995); R. N. Winn et al., *Marine Env. Res.* 40(3):247–265 (1995)).

As noted above, the invention is intended to further encompass progeny of a transgenic fish containing a genomically integrated bacteriophage lambda-derived transgene construct, as well as transgenic fish derived from a transgenic fish egg, sperm cell, embryo, or other cell containing a genomically integrated bacteriophage lambda-derived transgene construct. "Progeny", as the term is used herein, can result from breeding two transgenic fish of the invention, or from breeding a first transgenic fish of the invention to a second fish that is not a transgenic fish of the invention. In the latter case, the second fish can, for example, be a wild-type fish, a specialized strain of fish, a mutant fish, or another transgenic fish. The hybrid progeny of these matings have the benefits of the transgene for mutation detection combined with the benefits derived from these other lineages.

Definitions

An "expression vector" is a nucleic acid molecule containing a nucleotide sequence that is expressed in a host cell. Typically, the expression vector is a DNA molecule containing a gene, and expression of the gene is under the control of regulatory elements that can, but need not, include one or more constitutive or inducible promoters, tissue-specific regulatory elements, and enhancers. Such a gene or nucleic acid sequence is said to be "operably linked to" the regulatory elements.

A "cloning vector" is a nucleic acid molecule, typically a DNA molecule, that has the capability of replicating autonomously in a host cell. The cloning vector can, for example, be a plasmid, cosmid, or bacteriophage, and can be linear or circular. Cloning vectors typically contain one or more restriction endonuclease recognition sites at which foreign nucleic acid sequences can be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker sequence that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include nucleic acid sequences that encode polypeptides which can confer a phenotypic characteristic to the transformed cell, such as antibiotic resistence, test compound metabolism, and the like.

The terms "exogenous" or "heterologous," which are used interchangeably herein, denote some item, typically a nucleic acid sequence, that is foreign to its surroundings. In particular, the terms apply to nucleic acid sequences that have been inserted in to a host organism, but are not found in the normal genetic complement (i.e., genome) of the host organism. A gene that is heterologous with respect to an organism into which it has been inserted or transferred is sometimes referred to herein as a "transgene." A "transgenic" animal or host is an animal having one or more cells that contain exogenous (heterologous) nucleic acid sequences, including expression vectors. Although introduction of the heterologous nucleic acids into a host cell is not limited to any particular mode of delivery, microinjection of the heterologous DNA is preferred. Microinjection is labor-intensive and time-consuming, but when practiced in accordance with the present invention results in a greater likelihood of introducing the DNA into the cell nucleus as opposed to the cytoplasm. While advances have been made relating to other methods of gene transfer such as electroporation of eggs (e.g., K. Inoue et al., *Cell Differ. Dev.* 29(2):123–128 (1990); F. Muller et al., *FEBS Lett.*, 324 (1):27–32 (1993); Y. Murakami et al., *J. Biotechnol.*, 34 (1):35–42 (1994); F. Muller et al., *Mol. Mar. Biol. Biotechnol.* 1 (4–5):276–81 (1992)) and sperm (Symonds et al., 1994; D. A. Powers et al., *Mol. Mar. Biol. Biotechnol.*, 1 (4–5):301–8 (1992)); particle gun bombardment (A. V. Zelenin et al., *FEBS Lett.*, 287 (2):118–20 (1991)); liposzenes (J. Szelei et al., *Biochem. J.*, 259 (2):549–53 (1989)); and retroviral vectors (J. K. Lu et al., *Mol. Mar. Bio. Biotechnol.*, 6 (4):289–95 (1997)); these procedures only rarely result in transgene integration and germline transmission (K. Inoue et al., *Cell Differ. Dev.* 29(2):123–128 (1990)). Nonetheless, the invention is not intended to be limited to any particular method of introducing the heterologous DNA into the host organism. Preferably, the heterologous nucleic acid sequences are stably integrated into the host genome and are inheritable.

A "genomically identical" population of transgenic fish is one wherein each fish has the same genomic DNA as the others in the population; this can also be referred to as a clonal population. Members of a genomically identical population are produced by cloning or inbreeding within the population rather than by mating with non-genomically identical fish (see, e.g., D. C. Streisinger et al., *Nature*, 291:293–296 (1981); W. Driever et al., *Trends Genet.*, 10 (5): 152–159 (1994)). Zygosity of the genomically identical population can be haploid, diploid or triploid. A genomically identical population is a single sex (unisex) population and can be male or female. A genomically identical female population can be produced, for example, by gynogenesis, wherein sperm is used to activate the egg but does not contribute genomic DNA to the developing organism (J. H. Postlethwait et al., *Trends Genet.*, 13(5):183–190 (1997)). Methods for making a genomically identical male population include heat shock or irradiation.

A nucleotide sequence that is "excisable" from genomic DNA is one that can be isolated from the genomic DNA of the host animal, as by cutting the nucleotide sequence at one or more predetermined sites, for example at a lambda bacteriophage cos site. An "assayable" mutation target nucleic acid sequence is one wherein a mutated form of the nucleic acid sequence can be distinguished from the non-mutated form of the nucleic acid sequence, either directly or indirectly, as by using a laboratory assay or other detection procedure. Preferably, the presence or absence of a mutation in the nucleic acid sequence is detectable by way of a

chemical or biological assay. Detection can be mediated through the use of "reporter" nucleic acid sequences. For example, in a lac operon-based mutation detection system, a mutation in a lacI mutation target gene affects the expression of the lacZ reporter gene, and expression of the reporter gene is detectable in an *E. coli* host by assaying the ability of the host to produce the lacZ gene product (β-galactosidase) and thus metabolize a chromogenic substrate.

The term "mutagen" is to be broadly understood as meaning any mutagenic or potentially mutagenic agent or event, including a mutagenic chemical compound, such as a toxicant, or exposure to radiation, including but not limited to alpha, beta, or gamma emissions from an radioisotope, electromagnetic radiation of any frequency, such as x-ray, ultraviolet, or infrared radiation, exposure to an electromagnetic field (EMF), and the like.

Mutation Target Nucleic Acid Sequences

The assayable mutation target nucleic acid sequences present in the preferred transgenic fish of the invention are conveniently included in a bacteriophage λ shuttle vector. Use of a shuttle vector facilitates both introduction of DNA into the fish and recovery or rescue of the DNA from the fish. The shuttle vector preferably includes an excisable test nucleic acid sequence that contains at least one copy of at least one assayable mutation target nucleic acid sequence. The shuttle vector may, however, contain multiple copies of a particular target sequence, and/or two or more different assayable mutation target nucleic acid sequences.

In one preferred embodiment, the assayable mutation target nucleic acid sequence comprises at least one nucleotide sequence selected from the nucleotide sequences of the lac operon. Preferably, the assayable mutation target nucleic acid sequence is at least one of the lacI gene, the lacZ gene, or the lac promoter sequence. More preferably, the assayable mutation target nucleic acid sequence is the lacI gene.

In another preferred embodiment, the assayable mutation target nucleic acid sequence comprises at least one nucleotide sequence selected from the nucleotide sequences of the cII region of bacteriophage lambda. The cII region of bacteriophage lambda is approximately 300 base pairs in length and encompasses, among other sequences, the P_R promoter, the cII gene (which encodes the cII protein), the cII gene (which encodes the cII repressor protein), the cII MRNA ribosome binding site, and the cII protein-activated PRE promoter. Preferably, the assayable mutation target nucleic acid sequence is at least one of the cII gene, the cII mRNA ribosome binding site gene, or the P_{RE} promoter sequence. More preferably, the assayable mutation target nucleic acid sequence is the cII gene.

The nucleotide sequence of the bacteriophage lambda cII region, as disclosed in the Instruction Manual for the λ Select-cII Mutation Detection System for Big Blue Rodents (Catalog #7210210, Revision #028001, incorporated herein in its entirety) is set forth in FIG. 2A. The λ base positions (λ bp) displayed in the far left column are derived from the base positions of the wild-type lambda sequence (GenBank database accession numbers J02459, M17233, M24325, V00636 and X00906). The numbering convention used for the cII nucleotide positions (nt) listed in the second column is that of Schwarz et al. (*Nature*, 272: 410–414 (1978)). The positions of the cII PCR and sequencing primers used in the Stratagene mutation detection system are labeled. The cII primers used for PCR or sequencing of the cII gene in the alternative Epicentre Technologies mutation detection system are shown in FIG. 2B.

The cII protein activates transcriptional promoters in lambda that are essential for lysogenization in a bacterial host. Mutations in the cII region that lower the levels of cII protein result in a decreased ability of lambda to lysogenize. When grown under conditions that favor lysogeny in a suitable bacterial host cell, preferably an hfl⁻ *E. coli* strain, lambda prophages carrying such mutations survive only by the entering the lytic pathway of development, forming plaques. Prophages that are wild type for the cII region integrate into the bacterial host genome and become part of the developing bacterial lawn.

In a particularly preferred embodiment of the invention, the shuttle vector used to transfect the host fish cell is BIG BLUE λLIZ shuttle vector. This shuttle vector contains at least two mutation target regions: one derived from the lac operon, and the other comprising the lambda cII region. The packaging extract provided by Stratagene, Inc., recognizes the cos sites of the integrated lambda DNA and packages the sequences between the cos sites into phage particles. After the shuttle vector is recovered from the fish, the vector is packaged and introduced into an appropriate bacterial host *E. coli*, for example by using packaging extracts commercially available from Stratagene, Inc. (La Jolla, Calif.) or Epicentre Technologies (Madison, Wis.). Mutations in the lac region of the shuttle vector can be conveniently detected in a host *E. coli* cell using procedures described in the BIG BLUE Transgenic Rodents Mutagenesis Assay System Instruction Manual (Stratagene, La Jolla, Calif.). Alternatively or additionally, mutations in the cII region of the shuttle vector can be conveniently detected in a host *E. coli* cell using procedures described in either the λ Select-cII Mutation Detection System for BIG BLUE Rodents Instruction Manual (Stratagene, La Jolla, Calif.) or described in written instruction materials that accompany the Epicentre Technologies (Madison, Wis.) kit.

The λLIZ shuttle vector (FIG. 1) includes the *E. coli* lacI gene as a mutation target and alacZ as the reporter gene. The system uses a forward mutation assay capable of detecting a wide spectrum of mutations in the lacI target. The lacI target is the most well-characterized target gene for mutagenesis, with more than 30,000 mutants identified in prokaryotic and eukaryotic cells (see, e.g., S. W. Kohler et al., *Proc. Natl. Acad. Sci. U.S.A.* 88:7958–7962 (1991). Following mutagen exposure, genomic DNA is isolated from the tissues of interest. The shuttle vector is recovered or "rescued" from the genomic DNA by in vitro packaging, preferably by utilizing TRANSPACK packaging extract (Stratagene, Inc., La Jolla, Calif.) which packages the vector into viable phage particles. Mutations in lacI are detected histologically in *E. coli*, as described below. The BIG BLUE lac-based assay system has been shown to detect most classes of mutations, including base substitutions, single-base frameshifts, insertions, duplications, and deletions (J. C. Mirsalis et al., *Ann. Rev. Pharmacol. Toxicol.* 35:145–164 (1995); G. S. Provostet al., *Mutation Res.* 288:133–149 (1993)).

Mutations that interfere with the lac repression lead to transcription of the alacZ region, allowing formation of intact LacZ protein, β-galactosidase, through alpha complementation within the bacterial host. Mutations are analyzed by isolating genomic DNA from the transgenic animal and recovering the shuttle vector as functional bacteriophage using in vitro packaging. The packaging extract is preferably free of all known restriction systems (restriction minus) and permits efficient recovery of the phage independent of DNA methylation as is known in the art. The individually packaged phage infect and lyse *E. coli* bacterial hosts that produce complementing portions of the LacZ protein. In the presence of a chromogenic substrate, functional mutations in

lacI or the lac operator are seen as blue plaques on a lawn of clear nonmutant plaques. Mutant frequency is expressed as the ratio of the number of blue plaques to the total number of plaques tested. Each mutant can be further analyzed by sequence analysis of the lacI target gene without subcloning, using a modified polymerase chain reaction (PCR) method.

Alternatively, the cII gene in the λLIZ shuttle vector (FIG. 1) can serve as a mutation target. Selection of mutants in the λ Select-cII mutation detection system is based on the ability of bacteriophage λ to multiply through either the lytic or lysogenic cycle in *E. coli* host cells. The commitment to either lysis or lysogeny is made by lambda upon infection of an *E. coli* host cell and is regulated by a series of proteins, one of which is the product of the cII gene. Transcription of the cII gene is initiated at the P_R promoter, and the cII gene is transcribed as part of a polycistronic message, which includes cro, O, P, and Q. At two lambda promoters, the cII protein activates transcription of genes essential for the lysogenic response. One of these promoters is P_{RE}, which is located within the 5' end of the cII gene in the orientation opposite to that of cII transcription. P_{RE} establishes transcription of the gene for the cI repressor protein. The cI repressor protein binds to the O_R operator, inhibiting transcription at P_R and effectively shutting down transcription of several genes essential for the lytic response, committing the phage to the lysogenic cycle. In the λLIZ vector, the gene for the cI repressor protein contains the temperature-sensitive cI857 mutation that enables the cI repressor to be inactivated at 37° C., allowing titering in the preferred bacterial host strain (*E. coli* G1250). Thus, a detectable mutation in the cII gene either impairs the function of the cII protein or disables the P_{RE} promoter of the cI gene, in either event preventing transcription of the cI gene. In the absence of the cI repressor protein, the phage multiply through the lytic cycle. In contrast, a nonmutant cII gene results in a functional cI repressor protein, and the phage genome undergoes lysogenization.

Mutation Analysis

A mutated target sequence can be subjected to nucleic acid sequencing to determine the mutation spectrum characteristic of a particular mutagen, or of a particular tissue, or of the action of a particular mutagen on a particular tissue. A mutation spectrum reflects the frequency of certain specific types of mutations in a population of mutants. Types of mutations include, for example, nucleotide transitions (G/C to A/T and A/T to G/C), nucleotide transversions (G/C to T/A, G/C to C/G, A/T to T/A, and A/T to C/G), and frameshift mutations (e.g., +1, +2, -1 and -2). Alternatively, mutations can be identified using single nucleotide polymorphism analysis, or any other method known in the art for identifying or detecting single site mutations, insertions, deletions and frameshifts.

The mutation spectrum of a population of mutants can provide much useful information. In some instances, the spectrum is characteristic of a particular mutagen or class of mutagens, and can help identify the nature of the mutagenic compound. In other instances, a change in the mutation spectrum, relative to the mutation spectrum of a control group, may be evident even though the differences in mutation frequencies (experimental vs. control) are not statistically significant. In still other instances, mutation spectrum analysis can yield information about the sensitivity of different organs or tissues to a particular mutagen.

EXAMPLES

The following examples, while exemplary of the present invention, are not to be construed as specifically limiting the invention. Accordingly, variations and equivalents, now known or later developed, that would be within the purview of one skilled in the art are to considered to fall within the scope of this invention.

Example 1

Creation of a Transgenic Fish

The BIG BLUE Lambda Shuttle Vector λLIZ (Stratagene, Inc., La Jolla, Calif.; see the BIG BLUE Transgenic Rodent Mutagenesis Assay System Instruction Manual, specifically incorporated herein by reference, in its entirety) was used to create a transgenic medaka. Previous attempts to recover the λLIZ bacteriophage vector from both transgenic rodents and from fish that carry low gene copy numbers (e.g., 1–3 copies) have been problematic; it is highly desirable that transgenes are present at a level of several copies per cell or more to permit a high ratio of recovered target vector per genome (M. J. Dycaico et al., *Mutation Res.* 307:461–478 (1994)). Thus, efforts were made to increase the transgene copy number in the transgenic fish by creating a shuttle vector having multiple copies of the mutation target sequence. To this end, the λLIZ shuttle vector (about 50 kb) was cos-ligated, end to end, as linear concatamers. Specifically, the cos sites were melted by incubating λ DNA (50 μg) at 68° C. for 5 minutes, followed by ligation overnight (4° C.) with T4 ligase (0.5 mm ATP, 0.06 units/μl ligase, 1×ligase buffer, 714 ng/μl DNA) (New England Biolabs, Inc., Beverly, Mass.). DNA was brought up to 750 μl volume with STE (0.1 M NaCl, 10 mM Tris HCL, 1 mM ethylenediaminetetraacetic acid, EDTA, pH 8.0) and extracted twice with phenol/chloroform, once with chloroform, and precipitated with 100% ethanol. Following centrifugation, the pellet was washed with 70% ethanol, dried, and dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0) at a DNA concentration of 100–200 ng/μl, determined by conventional spectrophotometric technique. The resulting construct, possibly hundreds of kb in size, is orders of magnitude larger than other vectors (typically about 5 kb) successfully used to create transgenic fish, such as ψX174 (R. Winn et al., *Marine Environ. Res.*, 40:247–265 (1995)). Prior to microinjection, the DNA was diluted to a concentration between 50–100 ng/μl in ST.1E solution (5 mM Tris; 0.1 mM EDTA) and dialyzed on a filter (0.025 micron pore size, from Millipore, Bedford, Mass.) over ST.1E for 40 minutes. Relatively large amounts of DNA were used in each of the injections to increase the likelihood of genomic integration.

Initially, 490 one-cell stage medaka embryos were injected with heterologous DNA. Heterologous DNA was microinjected into one-cell stage medaka embryos substantially according to the method of Winn et al. (*Marine Environ. Res.*, 40(3):247–265 (1995), as modified herein. To maximize incorporation of the heterologous DNA and possibly reduce the degree of mosaicism in the founders, fertilized eggs at the one-cell stage were collected by removing egg masses from the vent of the female fish beginning 2 hours prior to the onset of a 16-hour light cycle and every 10–15 minutes thereafter. The gene transfer method was optimized by rigidly controlling the timing of the injection at the earliest 1-cell stage of development of the fish, in most cases within 5 minutes of fertilization. The embryos were individually separated by removing the entangling chorionic fibrils and examined to verify the one-cell stage of development for efficient gene transfer. The one-cell embryos were placed within a watch-glass filled with 18‰ (parts per thousand salinity) seawater to better visualize the penetration of the injection needle and to reduce the incidence of fungal infection.

Injection of the heterologous DNA was performed with the aid of a dissection microscope, micromanipulators, and an N_2 pressurized gas injection apparatus (model PLI100 commercially available from Medical Systems Corp., Greenvale, N.Y.). The embryo was held in place with a capillary pipette (25 μm) secured with a micromanipulator. Another capillary pipette pulled to a fine tip (1–2 μm) secured by a micromanipulator, and attached to a gas injection apparatus served to inject the embryo. The DNA solution was injected through a continuously flowing pipette into the cytoplasm of the one-cell embryo, or through the micropyle if visible. This is in contrast to the technique used in rodents, wherein the DNA solution can be directly injected into the rodent cell nucleus. It is believed that injection through the micropyle is preferable since introduction into the cytoplasm may increase the likelihood of degradation of the DNA construct and, more important, may give rise to mosaic (or chimeric) integration of the gene in the tissues of a resultant transgenic fish. That is, not all of the cells will have the heterologous DNA integrated chromosomally. Mosaic integration of the transgene in transgenic fishes is very common and is problematic because germ-line transmission is not guaranteed even if DNA extracted from a fin clip (the typical assay for integration) indicates the founder fish carries the gene. The flow rate and the total amount of solution injected was controlled by adjusting the pressure of the gas and the duration of the injection which permitted injection of approximately 5–20 nl DNA solution.

Injected embryos were transferred into dechlorinated tap water and incubated therein using several different procedures. Some embryos were placed on 20 mm petri dishes, others were placed on cell culture plates, and others were placed in containers with air supply ("bubblers") to allow for constant aeration during growth at 26° C. until hatching (about 10 to 12 days). Embryos were examined daily and any dead were removed from the dishes. An earlier attempt was made that involved incubation in Yamamoto's solution (Yamamoto, "Medaka (killifish): Biology and Strains," Keigaku Publishing Co., Tokyo, Japan, 1975) for 5 days, and then in sterile culture water until hatching in approximately 10 days, however this procedure was discontinued after reduced survival in this solution was observed. A total of 141 fish survived in this initial experiment to age 6–8 weeks (28%) and were analyzed for the presence of heterologous DNA as described below. In a second experiment, a total of 238 fish (4–6 weeks post hatch) were analyzed in the same way.

Genetic screening for the presence of integrated λLIZ sequences and gene copy number evaluation of F_1 generation fish were simultaneously performed via PCR using a new instrument and DNA detection system. This accelerated the assessment of the positive F_1 generation fish which were not mosaic for integration of the transgene and therefore permitted the establishment of the specific high copy number lineages. The traditional method of copy number quantitation using Southern blot methods (described below) was not necessary.

Enhancements in the PCR method over the last decade have fostered the development of numerous and diverse applications. A recent enhancement facilitates real-time, quantitative analysis of the DNA amplification using fluorescence-based PCR. The analysis uses a DNA sequence detection instrument (ABI PRISM 7700 Sequence Detection System, Perkin Elmer Applied Biosystems, Foster City, Calif., USA) which combines PCR with a fluorogenic 5' nuclease assay. Pairs of primers (forward and reverse) 18–25 mer oligonucleotides that anneal within the DNA sequence of interest are synthesized in order to generate an amplified product generally ranging from 60–70 base pairs (bps) in size. A 20–30 mer oligonucleotide is synthesized as a probe to anneal within the PCR product generated by the forward and reverse primers. This fluorogenic, target-specific probe comprises an oligonucleotide with both a reporter and a quencher dye attached, and anneals to the target sequence between the forward and reverse primers. The probe typically includes a FAM (6-carboxy-fluorescein) reporter dye linked to the 5' end of the oligonucleotide and TAMRA (6-carboxy-tetramethylrhodamine) attached at the 3' end of the oligonucleotide as the quencher dye. As the PCR proceeds the probe is cleaved by the 5' nuclease activity of a DNA polymerase which permits the detection of the reporter dye signal. The DNA sequence-specific fluorescence signal is generated and detected in solution during the PCR. Assuming a 100% efficiency of amplification, the threshold cycle decreased by one cycle as the concentration of the template doubles. Therefore, the input DNA target quantity can be determined based on the cycle at which fluorescent signal is first detectable. The quantitative PCR analysis incorporates kinetic analysis in which the initial copy numbers of unknowns are determined by comparison with a curve generated from samples of known initial DNA quantities.

The typical conditions for the fluorescence-based PCR were as follows: 100 ng DNA; 4 mM $MgSO_4$; 200 M dATP, dCTP, dGTP; 400 uM dUTP; 125 M probe; 0.625 U Amplitaq Gold™ polymerase; 0.25 U Amerase UNG; and 1× of supplied buffer. A two stage amplification profile was used with 2 minutes at 50° C., 10 minutes at 95° C.; followed by stage two with 15 seconds at 95° C., 1 minute at 60° C. for a total of 30 cycles. A hold cycle at 25° C. was used following completion of amplification. Samples were prepared using sterile water in the place of DNA in the reagent mixture as no template DNA controls. Replicate serial dilutions of known quantities of DNA, were prepared to construct a standard curve from which the quantities of unknown samples were estimated.

In this example, screening for the presence of integrated λLIZ was accomplished prior to copy number determination using a standard PCR assay on DNA isolated from excised fin tissue. Genomic DNA is extracted from caudal fin tissue excised from presumptive transgenic fish aged 1–2 months. The DNA is extracted by homogenization of the tissue in 0.3 mL 1×SSC (150 mM NaCl, 15 mM sodium citrate; see page B.13 of Sambrook et al., Molecular Cloning, a Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989), 0.5% sodium dodecyl sulfate, SDS, and 10 mg/mL proteinase K for 3 hours at 55° C. Samples were extracted twice with methylene chloride:isoamyl alcohol (24:1) containing 0.1 5 M NaCl, precipitated with two volumes 100% ethanol and resuspended in TE buffer (10 mM Tris, 1 mM EDTA pH 8.0). The DNA concentration is then estimated by measurement of absorbence at 260 nm. Several lacI sequencing primer pairs (commercially available under the trade designation BIG BLUE lacI PCR Primer Set, from Stratagene, La Jolla, Calif.) can be used to generate PCR products of about 200–800 kb. A standard amplification temperature profile is: initial denaturation 95° C., 60 seconds; denaturation 95° C., 30 seconds; annealing 60° C., 30 seconds; and extension 72° C., 60 seconds. When using this screening method, we utilized two oligonucleotide primers, "Lambda 1" (5'-GAT GAG TTC GTG TCC GTA CAA CTG G, SEQ ID NO:4) and "Lambda 2" (5'-GGT TAT CGA AAT CAG CCA CAG CGC C-3', SEQ ID NO:5), and PCR conditions of initial denaturation 94° C., 30 seconds; dena-

turation 60° C., 30 seconds; extension 72° C., 60 seconds, for 30 cycles. Electrophoresis of the PCR products on an agarose gel (1–2%) can be used to confirm amplification of the DNA fragment of appropriate size. In previous experiments, integration rate in founder (F_0) transgenic medaka has typically been approximately 7–10%; however using the procedures set forth in this example, the integration rate jumped to 16%.

In this alternative method, copy number determination can be accomplished using a Southern blot hybridization using a biotinylated λLIZ DNA probe and chemiluminescent detection. Genomic DNA (5–10 μg) from previously PCR-screened positive transgenic fish is digested with HindIII in the presence of 4 mM spermidine to improve digestion, followed by electrophoresis on a 0.8% agarose gel and then transfer to a nylon membrane using conventional biotechnological techniques. The membrane is baked at 80° C. for 30 minutes and exposed to a UV transilluminator for 5 minutes. Prehybridization is performed at 42° C. for 1 hour in a 20 mM sodium phosphate buffer (pH 7.5) containing 50% formamide, 5×SSC, 5×Denhardt's solution (Sigma Chemical Company, St. Louis, Mo.), 0.1% SDS and 100 μg/mL denatured calf thymus DNA. Hybridization is performed at 42° C. for 16 hours with about 20 ng of a biotinylated λLIZ DNA probe. Membranes are washed twice for 5 minutes at 20° C. in 2×SSC, 0.1% SDS, and twice for 15 min at 68° C. in 0.2×SSC, 0.1% SDS. Membranes are analyzed by chemiluminescent detection according to the manufacturer's detection protocol with film exposures of 20–60 minutes (commercially available under the trade designation PHOTOTOPE CHEMILUMINESCENT DNA DETECTION KIT, from New England Biolabs, Beverly, Mass.). Copy number standards, prepared by adding λLIZ DNA (equivalent to 1–50 copies per genome) to calf thymus DNA (5 μg), are loaded adjacent to the DNA from presumptive transgenic fish. Copy number estimates can be made by using the DNA content of 2.2 pg for the diploid genome of medaka.

From the initial experiment, 9 of the 141 founders were shown to have incorporated the μLIZ vector, and of these 9, two showed germline transmission. Likewise, in the second experiment, 53 of the 238 founders were shown to have incorporated the vector, and 13 of the 53 showed germline transmission. Together, a total of 62 fish (16% of the founder population) showed positive amplification of the λLIZ product of the appropriate size (~50 bp), and 15 of those showed germline transmission (24%). The transgenic founders that showed germline transmission when mated with wild-type mates transmitted the DNA sequence to their offspring at frequencies of 3–40% (Table 1). The observed gene transfer frequency represents an improvement over previous efforts, and ranks among the highest reported in other transgenic fish efforts. The variable frequencies of gene transmission to F_1 offspring demonstrated the mosaic germ-line integration characteristic of transgenic fish.

Gene copy number results for the selected members of this transgenic founder population are also shown in Table 1. Of eleven transgenic lineages analyzed, five lineages carry 2 copies, including one lineage (#310) that carries in excess of 75 copies.

TABLE I

Estimated gene copy number and frequency of germ-line transmission for selected transgenic λLIZ medaka lineages

Founder lineage frequency	Copy number	Germ-line transmission
013	1	35%
108	1	3%
188	1	20%
203	~4–5	13%
254	1	40%
261	~3–4	20%
304	1	36%
310	>75	12%
327	~1	5%
361	~2	10%
370	~2	29%

Following confirmation of positive integration of λLIZ in the F_1 generation, the transgenic siblings were bred to produce F_2 generation fish. The quantitative fluorescence-based PCR procedures described above facilitated distinguishing the homozygous and hemizygous transgenic F_2 generation fish, thereby speeding the establishment of homozygous lines by eliminating the need to perform time-consuming traditional methods that rely on DNA hybridization techniques (i.e. Southern blots). Homozygous status was verified genetically by crossing presumptive homozygous fish with wild-type fish, in which case the presence of 100% positive transgenic offspring confirmed homozygous status. In addition, by using these quantitative fluorescence-based PCR procedures, transgenic lineages that carry multiple copies of the vector, and therefore have the greatest promise for efficient recovery of the vector, were identified.

These results compare favorably to those obtained from producing transgenic rats with the identical mutation target. In this effort, 62 transgenic founders were obtained out of a total of 379 injected embryos that survived to an age of 6–8 weeks, or 16%. For the transgenic rat study, over 12,000 Fisher 344 inbred rat embryo injections produced 257 surviving pups and resulted in 17 transgenic founders, or 6.6% (M. J. Dycaico et al., *Mutation Res.* 307:461–478 (1994)). Although the majority of these 17 rodent founders carried less than 5 copies of λLIZ, two founders carried more than 20 copies, such that homozygous individuals of subsequent generations carried at least 40 copies. Attempts to recover the vector from λLIZ transgenic rats that carried only 1–2 gene copies was not possible in several lineages, and, in other low copy number lineages from which recovery was possible, the low rescue efficiencies increased the time and costs of analyses as compared to those for higher copy number animals.

Example 2

Recovery of λLIZ Shuttle Vector

Fish tissues appear to pose a significant problem related to efficient recovery of bacteriophage and plasmid-based vectors for mutation detection. The extraction of high quality and high molecular weight genomic DNA is very important to the efficient recovery of the shuttle vector from transgenic rodent tissues. However, repeated attempts to recover shuttle vectors from transgenic fish tissues using the procedures developed for rodents have in the past been unsuccessful.

Isolation of Genomic DNA

Initial recovery of the shuttle vector was attempted using DNA obtained from two homozygous fish lineages that carried only ~1–2 copies of the vector (lines #13 and 108, Table I). The vector recoveries from these fish were unsuc-

cessful. Vector recovery from animals which carry low copy numbers has previously been shown to be problematic in transgenic rodent studies. Therefore, transgenic fish that had relatively high gene copy numbers (>5–10 copies) and demonstrated stable germ-line transmission were selected for the analysis of recovery and spontaneous mutation frequency of λLIZ. In addition, standard procedures for isolating genomic DNA from rodents were altered so as to insure isolation of high quality genomic DNA sufficient for recovering shuttle vectors from the fish, as described below.

Transgenic F_1 generation fish (4–6 weeks old, line #310, hemizygous for ~100 copies) were either flash frozen in liquid nitrogen or quickly minced prior to placing in a dounce homogenizer containing 2 mL douncing buffer (1×SSC, 1% SDS). Fish were dounced twice only and transferred to a centrifuge tube. An additional 3 mL of dounce buffer was added to the centrifuge tube, and freshly made Proteinase K solution (150 μL, 20 mg/mL) was then added as well. Samples were placed at 37° C. for 1½ hours. Tubes were inverted two times at 30 minute intervals. Samples were extracted with 5 mL 50:50 buffered phenol:chloroform by inverting 5 times followed by centrifugation at 4000 rpm for 10 minutes. Supernatant was placed in a clean centrifuge tube and the phenol:chloroform extraction, including the centrifugation, was repeated. The supernatant was again removed, and 8M potassium acetate was added thereto to a final concentration of 1M. An equal volume of chloroform was also added, the tubes were inverted gently, and were then centrifuged as before. Supernatant was removed to a clean tube to which two volumes of 100% ethanol were added. Tubes were inverted gently several times and allowed to sit at room temperature (about 22° C.) for 10 minutes. Precipitated DNA was spooled with a flame-sealed Pasteur Pipette, dried in air, and resuspended in 50–100 μL TE buffer (Tris 10 mM, EDTA, 1 mM, pH 7.5). Importantly, this protocol incorporates a shorter digestion time (1½ hours) at a lower temperature (37° C.) compared to the standard procedure used to isolate genomic DNA from mice (wherein the digestion is carried out at 50° C. to 55° C. for 3 hours), thus enhancing the recovery of assayable genomic DNA from the fish.

It should be noted that this DNA extraction method can be used to recover DNA directly from an organ or a tissue of a fish. The protocol is typically carried out using reduced volumes (since the amount of biological material to be extracted is reduced), and the ethanol-precipitated DNA can be isolated by centrifugation rather than by spooling. This method offers great benefit in that it allows for the study of tissue-specific mutation frequencies.

Packaging Reaction

From these genomic fish DNAs, the λLIZ vector was recovered and packaged into empty phage particles creating infective phage according to the protocols recommended by the manufacturer for in vitro packaging (TRANSPACK in vitro packaging extract, Stratagene, La Jolla, Calif.). Specifically, the packaging extract was added to 8 μl of genomic DNA (>0.5 mg/mL), incubated at 30° C. for 90 minutes, at which time the packaging reaction was terminated by adding SM buffer.

Infection of Bacterial Host and Screening: λ—Select cII Assay

The individually packaged lambda phage were used to infect the *E. coli* host strain G1250 (see FIG. 3A). The G1250 plating cell culture was mixed with diluted (titer) and undiluted packaged DNA (mutant screening). Titer plates were placed at 37° C. overnight, and screening plates were placed at 24° C. for 40 hours. A 40 hour incubation is preferred to the recommended 48 hour incubation to reduce mottling on the plates. Because the λLIZ shuttle vector contains the temperature-sensitive cI857 mutation, λ$^+$ multiply through the lytic cycle under the nonselective conditions, resulting in plaque formation. The rescue efficiency was determined by averaging the number of plaques on the three titer (dilution) trays and computing the total number of plaques obtained per packaging reaction. The spontaneous mutation frequency was calculated by dividing the number of confirmed mutants by the estimated total number of plaques recovered.

An average of 2,060,000±65,000 plaques were recovered from a single packaging reaction of line 310 hemizygous fish, indicating very high efficiency in the recovery of the phage. Table II shows the relationship between copy number and phage recovery, showing that attempts to recover the phage from low copy number lineages were not successful. Recovery was achieved from lines 203 and 310, with line 310 exhibiting exceptional efficiency, particularly in comparison to the 625,000 plaques recovered from Big Blue mouse DNA analyzed simultaneously. A minimum of 300,000 plaques is recommended for statistical purposes.

TABLE II

Relationship of copy number and efficiency of λ recovery

Founder lineage	Copy number	Recovery (pfu)
13	1	none
108	1	none
188	1	(1)
203	4–5	0.05×10^6
254	1	(1)
261	3–4	none
304	1	(1)
310	>75	$3\text{-}16 \times 10^6$
370	2	none

(1) Phage rescue was not attempted from these samples.

Infection of Bacterial Host and Screening—lac Assay

Individually packaged lambda phage from four of the fish (lineage 310) were absorbed onto *E. coli* SCS-8 cells (commercially available from Stratagene), mixed with top agarose (NZY medium) with 0.7% agarose) containing (1.5 mg/mL) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and plated onto 25×25 cm square bioassay trays containing 250 mL NZY bottom agar (NZY medium with 1.5% agar) (see FIG. 3B). Packaged control λ were plated on VCS-257 cells using serial dilutions. Alternatively, the absorbed phage mixture can be sampled, diluted and plated in duplicate or triplicate as plating efficiency plates to determine the total number of phage per packaging reaction kit. The number of plates per packaging reaction was adjusted to give a target packaging density of about 15,000 plaques per plate (24 plaques/cm^2). Experiments included color control plates using a series of lacI mutants that produce a spectrum of color intensities from very faint (CM-0) to intense dark blue (CM-3). Plates were incubated for 16–18 hours at 37° C.

After incubation, plates were visually screened for blue mutant plaques using a red enhancement screening filter. The total number of plaques was counted on plating efficiency plates to calculate total plaques screened. The efficiency of the packaging extract was determined by counting the plaques on control λ plate. Bacterial lawn and plaques are blue because VCS257 contains and expresses the intact lacZ gene.

The efficiency of the packaging extract was determined by:

$$\frac{(\#\text{ of plaques}) X\,(\text{dilution factor}) \times (\text{total packaging volume})}{(\mu g\ \text{control}\ \lambda) \times (\mu g\ \text{plated})} = \text{pfu/mg DNA}$$

and was expressed as plaque forming units (pfu) per mg DNA.

The rescue efficiency was determined by averaging the number of plaques on the dilution trays and computing the total number of plaques obtained per packaging reaction. A total of over 500,000 pfu were recovered from each of the four samples. The efficiency of the recovery can be evaluated and related to the amount of DNA necessary to obtain a recommended 300,000–500,000 plaque forming units (pfu)/analysis.

Example 3

Mutagen Exposure Experiments

Mutagens

N-ethyl-N-nitrosourea (ENU) is a well-characterized mutagen and carcinogen that acts by direct ethylation of oxygen and nitrogen in the bases of DNA (B. Singer, *Nature* 264:333–339 (1976); B. Singer et al., *Nature* 276:85–88 (1978)). ENU is a useful agent for the study of the relationship of mutation to DNA repair, replication, adduct persistence, and cell differentiation (J. G. Burkhart et al., *Mutation Res.* 292:69–81 (1993)). A limited study of ENU-induced mutation has been previously performed using medaka (A. Shimada et al., *Zoological Sci. (Tokyo)* 8(6) :1127 (1991); A. Shimada et al., *Zoological Sci. (Tokyo)* 7(6):1053 (1990)).

Dimethylnitrosamine (DMN) is a methylating agent and potent liver carcinogen in mice (International Agency for Research on Cancer (IARC), IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 7, IARC, Lyon. 253p (1974)). Since cell proliferation is an important parameter for the induction of mutations, it is important to consider the influence of fixation time of adducts on the induction of mutations. DMN has been used as a representative mutagen which forms methylated DNA adducts in transgenic mice (J. C. Mirsalis et al., *Mutagenesis* 8:265–271 (1993)). DMN is among the nitrosamines that have induced hepatocarcinogenesis in fish with progressive stages similar to those characterized in rodent hepatic neoplasia (W. E. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor and Francis. 421–446 (1995)).

Exemplary Exposure Regimen

Fish mutagen exposure is performed by using protocols employed in previous transgenic rodent studies (e.g., M. J. Dycaico et al., *Mutation Res.* 307:461–478 (1994); B. J. Rogers et al., *Mutation Res.* 327:57–66 (1995)). Specifically, one regimen consists of a single-pulse 4 hour exposure; another consists of a multiple-pulse exposure regimen consisting of 2 pulses for a 4 hour exposure for 7 days. Prior to final exposure, range-finding assays for each of the two model compounds are conducted wherein fish are exposed in single-pulse and multiple-pulse treatments over a range of mutagen concentrations to determine the upper exposure concentration expected to produce minimal or zero lethality (about 50% of the lowest exposure concentration at which deaths occur). In previous mutagen exposure studies using λLIZ transgenic mice, doses of 50–250 mg/kg of ENU (LD_{50}~350 mg/kg) were used to obtain at least a two-fold mutation frequency induction. The range finding trials in the present example are 0, 25, 50 and 100 ppm of ENU and DMN.

Fish are placed in replicated 40 mL acid-washed borosilicate glass test chambers (20/chamber). The mutagen solutions, at concentrations determined by dilution factors, are added to water immediately prior to the initiation of exposure. Toxicant-free treatments accompany all exposures as controls. For the multiple-pulse regimen, fish are transferred and held in clean water to await the next exposure. The fish are not fed during the exposure period. During the exposure phase, fish are monitored regularly for any visual signs of distress. Any dead or moribund fish are removed.

Following the final exposure series, fish are rinsed and transferred to grow-out aquaria for a prescribed expression time. During this time fish are held in aquaria in toxicant-free water at 26° C. on a 12:12 hour light:dark regime and fed twice daily. The fish are visually monitored at least twice daily during feeding. Any fish that have died, or that exhibit abnormal swimming behaviors or other visible signs of distress, are be removed from the aquaria. Fish that show apparent formation of external neoplasms are removed, sacrificed, and saved for further analyses, if desired.

The influence of expression time on the mutation frequency is evaluated by sampling fish at 5, 10, and 15 days following exposure. Expression time, or fixation time, is defined as the time allowed between dosing and sacrificing the animals for mutation assays. Some expression time is required, especially after single-dose administrations, to permit uptake and distribution of the chemical, metabolic activation to a DNA-reactive form, formation of adducts, and at least one cell division to "fix" the adduct as a heritable mutation. Although there is no data currently available on mutations rates, DNA repair, or cell proliferation in transgenic medaka, it is believed that a long expression time (>7 days) would allow adequate time to either repair DNA adducts or fix adducts as mutations. This reduces the possibility the DNA adducts will be mutated by the host *E. coli* by decreasing the number of DNA adducts present on the recovered target DNA.

ENU Exposure and Analysis

Medaka (50 hemizygous male and female, 4–8 weeks old) were exposed to 100 ppm and 200 ppm ENU for 1 hour in dechlorinated tap water in replicate 500 mL beakers. They were then transferred to clean water (no ENU) and held for 15 days. At that point the fish were quickly sacrificed using an overdose of MS-222 (tricaine methanesulfonate), flash frozen in liquid nitrogen, and stored at −70° C.

Mutagenesis in experimental and control fish was analyzed using the cII assay system as described in Example 2. The results in Table III show a greater than two-fold increase in cII mutations in the 100 ppm-exposed fish compared to the control fish, and an induced mutation frequency in the 200 ppm-exposed fish of nearly four times that of the controls. The overall spontaneous mutation frequency was determined to be $2.92 \pm 0.68 \times 10^{-5}$ for the cII target.

TABLE III

N-Nitroso-N-Ethylurea (ENU) Exposure Summary

	Recovery ($\times 10^6$) pfu	Number of Mutants	Mutation Frequency ($\times 10^{-5}$)
Controls	3.03	97	3.20
	2.37	58	2.45
	1.62	58	3.59
	1.91	64	3.35
	2.38	42	1.76
	2.4	76	3.17
			$X = 2.92 \pm 0.68$

TABLE III-continued

N-Nitroso-N-Ethylurea (ENU) Exposure Summary			
	Recovery ($\times 10^6$) pfu	Number of Mutants	Mutation Frequency ($\times 10^{-5}$)
100 ppm	1.79	129	7.21
	2.31	118	5.11
	1.64	125	7.65
	1.70	114	6.73
	2.48	243	9.80
	1.28	149	11.67
			X = 8.03 ± 2.34
200 ppm	1.79	159	8.88
	3.95	399	10.10
	1.56	133	8.55
	1.73	198	11.48
	1.60	341	21.27
	1.54	176	11.45
			X = 11.96 ± 4.7

Similar results (a two-fold increase) were observed for the lacI target in the 100 ppm-exposed fish. A spontaneous mutation frequency for the lacI target was calculated by dividing the number of confirmed mutants (blue plaques) by the estimated total clear plaques, and determined to be about 1.01×10^{-5}. For comparison, the spontaneous mutation frequency of the identical λLIZ mutation target in transgenic mice and rat tissues has been determined to be $2–4\times10^{-5}$ (G. S. Provost et al., *Mutation Res.* 288:133–149 (1993); M. J. Dycaico et al., *Mutation Res.* 307:461–478 (1994)).

Example 4

Mutation Analysis of the cII Target Gene

Putative mutant plaques were verified by coring and re-plating them with *E. coli* G1250 to confirm phenotypic stability of the mutant. Selected mutations were analyzed by sequence analysis of the cII target gene using linear amplification sequencing in which the components of a chain-termination sequencing reaction were cycled through a standard PCR temperature profile as described in the λ SELECT-cII Mutation Detection System for BIG BLUE Rodents instruction manual, Catalog #72010, Revision #028001. The cII target region from a purified λcII mutant was amplified by PCR using λ SELECT-cII sequencing primers, commercially available from Stratagene, La Jolla, Calif. Briefly, a plaque was transferred to a microcentrifuge tube containing 25 μL autoclaved dH_2O. The tube was capped securely and placed in boiling water for 5 minutes, then centrifuged at maximum speed for 3 minutes. The supernatant (10 μL) was immediately transferred to 40 μL of a PCR mastermix such that the final concentrations of the reagents were 1×Taq polymerase reaction buffer, 10 pmol of each primer, 12.5 nmol of each dNTP, and 2.5 U of Taq2000 DNA polymerase. The amplification reaction was overlayed with a drop of sterile mineral oil and the template was amplified using the following cycling parameters: a 3 minute denaturation at 95° C., followed by 30 cycles of 30 seconds at 95° C., 1 minute at 60° C., and 1 minute at 72° C., with a final extension of 10 minutes at 72° C. The products (2–4 μl) were loaded on a sequencing gel and analyzed for sequence differences; the results are shown in Table IV.

TABLE IV

Mutation	% Spontaneous	ENU induced %
Transitions		
G/C → A/T	25	25
A/T → G/C	15	24
Transversions		
G/C → T/A	20	14
G/C → C/G	10	14
A/T → T/A	5	19
A/T → C/G	0	0
Frameshift (−1)	20	5
(+1)	5	0

Example 5

Mutation Analysis of the lacI Target Gene

Putative mutant plaques can be verified by coring and re-plating them with *E. coli* SCS-8 on X-gal-containing medium to confirm phenotypic stability of the mutant. Selected mutations can be analyzed further by sequence analyses of the lacI target gene using the linear amplification sequencing which is a variation of the polymerase chain reaction (commercially available from Stratagene, Inc., La Jolla, Calif.) under the trade designation BIG BLUE CYCLIST Exo-Pfu). The components of a chain-termination sequencing reaction are cycled through a temperature profile consisting of a heat-denaturation step, an annealing step and an extension step, as described below.

Mutant plaques can be verified and isolated from non-mutant plaques re-plating in the presence of X-gal. LacI template DNA can be prepared for sequencing by PCR using the mutant λ phage directly as the template or by further purification (See Gu et al. *Mutation Res.*, 307:533–540 (1994)). The sequencing reaction mixture can be prepared as follows: 200 fmol template DNA, 1 pmol of primer (commercially available under the trade designation of BIG BLUE lacI PCR Primer Set, from Stratagene), 4 μl of 10×sequencing buffer, 10 μCi of radioactive label, 1 μl (2.5 U) of polymerase, water to 26 μl and 4 μl DMSO to a final volume of 30 μl. The mixture is cycled through a temperature profile of denaturation 95° C., 5 minutes; annealing 60° C., 30 seconds; and extension 72° C., 60 seconds. The products (2–4 μl) are loaded on a sequencing gel and analyzed for sequence differences. The sequence information on lacI as a target for mutagenesis suggests that many of the sites for inactivation of the lacI have been identified. Comparisons can be made with the type, number and percentage of mutations identified.

Sequence Listing Free Text

SEQ ID NO: 1	Portion of the BIG BLUE λLIZ Shuttle Vector, Stratagene, La Jolla, CA
SEQ ID NO: 2	cII protein
SEQ ID NO: 3	cII gene
SEQ ID NO: 4	Oligonucleotide
SEQ ID NO: 5	Oligonucleotide

The complete disclosures of all patents, patent applications, publications, database entries, submissions and deposits, including GENBANK deposits and the descriptive information associated therewith, and other documents cited herein are fully incorporated herein in their entireties by reference as if individually incorporated. Various modifications and alterations of this invention will become apparent

to those skilled in the art without departing from the scope and spirit of this invention, and it should be understood that this invention is not to be undnly limited to the illustrative embodiments set forth herein.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 5

<210> SEQ ID NO 1
<211> LENGTH: 918
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Portion of Stratagene BIG BLUE lambda LIZ shuttle vector

<400> SEQUENCE: 1

tccccatctt gtctgcgaca gattcctggg ataagccaag ttcatttttc ttttttttcat 60

aaattgcttt aaggcgacgt gcgtcctcaa gctgctcttg tgttaatggt ttctttttgg 120

tgctcatacg ttaaatctat caccgcaacg gataaatatc taacaccgtg cgtgttgact 180

attttacctc tggcggtgat aatggttgca tgtactaagg aggttgtatg gaacaacgca 240

taaccctgaa agattatgca atgcgctttg ggcaaaccaa gacagctaaa gatctcggcg 300

tatatcaaag cgcgatcaac aaggccattc atgcaggccg aaagattttt ttaactataa 360

acgctgatgg aagcgtttat gcggaagagg taaagccctt cccgagtaac aaaaaaacaa 420

cagcataaat aaccccgctc ttacacattc cagccctgaa aaagggcatc aaattaaacc 480

acacctatgg tgtatgcatt tatttgcata cattcaatca attgttatct aaggaaatac 540

ttacatatgg ttcgtgcaaa caaacgcaac gaggctctac gaatcgagag tgcgttgctt 600

aacaaaatcg caatgcttgg aactgagaag acagcggaag ctgtgggcgt tgataagtcg 660

cagatcagca ggtggaagag ggactggatt ccaaagttct caatgctgct tgctgttctt 720

gaatgggggg tcgttgacga cgacatggct cgattggcgc gacaagttgc tgcgattctc 780

accaataaaa aacgcccggc ggcaaccgag cgttctgaac aaatccagat ggagttctga 840

ggtcattact ggatctatca acaggagtca ttatgacaaa tacagcaaaa atactcaact 900

tcggcagagc taactttg 918

<210> SEQ ID NO 2
<211> LENGTH: 97
<212> TYPE: PRT
<213> ORGANISM: Escherichia coli

<400> SEQUENCE: 2

Met Val Arg Ala Asn Lys Arg Asn Glu Ala Leu Arg Ile Glu Ser Ala
1 5 10 15

Leu Leu Asn Lys Ile Ala Met Leu Gly Thr Glu Lys Thr Ala Glu Ala
20 25 30

Val Gly Val Asp Lys Ser Gln Ile Ser Arg Trp Lys Arg Asp Trp Ile
35 40 45

Pro Lys Phe Ser Met Leu Leu Ala Val Leu Glu Trp Gly Val Val Asp
50 55 60

Asp Asp Met Ala Arg Leu Ala Arg Gln Val Ala Ala Ile Leu Thr Asn
65 70 75 80

Lys Lys Arg Pro Ala Ala Thr Glu Arg Ser Glu Glu Ile Gln Met Glu
85 90 95

Phe

-continued

<210> SEQ ID NO 3
<211> LENGTH: 446
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: cII gene

<400> SEQUENCE: 3

aaaaagggca tcaaattaaa ccacacctat ggtgtatgca tttatttgca tacattcaat 60

caattgttat ctaaggaaat acttacatat ggttcgtgca aacaaacgca acgaggctct 120

acgaatcgag agtgcgttgc ttaacaaaat cgcaatgctt ggaactgaga agacagcgga 180

agctgtgggc gttgataagt cgcagatcag caggtggaag agggactgga ttccaaagtt 240

ctcaatgctg cttgctgttc ttgaatgggg ggtcgttgac gacgacatgg ctcgattggc 300

gcgacaagtt gctgcgattc tcaccaataa aaaacgcccg gcggcaaccg agcgttctga 360

acaaatccag atggagttct gaggtcatta ctggatctat caacaggagt cattatgaca 420

aatacagcaa aaatactcaa cttcgg 446

<210> SEQ ID NO 4
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
oligonucleotide

<400> SEQUENCE: 4

gatgagttcg tgtccgtaca actgg 25

<210> SEQ ID NO 5
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
oligonucleotide

<400> SEQUENCE: 5

ggttatcgaa atcagccaca gcgcc 25

What is claimed is:

1. A transgenic fish whose genomic DNA comprises a recoverable assayable mutation target nucleic acid sequence operably linked to a bacteriophage lambda-derived transgene construct; wherein the mutation target nucleic acid sequence is recoverable from the transgenic fish, transferable into a bacterial host, and assayable in the bacterial host for the presence of a mutation.

2. The transgenic fish of claim **1** wherein the assayable mutation target nucleic acid sequence comprises at least one nucleic acid sequence selected from the group consisting of the lacI gene, the lacZ gene, the lac promoter sequence, the cII gene, the cII mRNA ribosome binding site and the cII protein-activated P_{RE} promoter sequence.

3. The transgenic fish of claim **2** wherein the assayable mutation target sequence comprises the lacI gene.

4. The transgenic fish of claim **2** wherein the assayable mutation target sequence comprises the cII gene.

5. The transgenic fish of claim **1** wherein the assayable mutation target sequence comprises the cII region of bacteriophage lambda.

6. The transgenic fish of claim **1** wherein the bacteriophage lambda-derived transgenic construct comprises at least one cos site.

7. The transgenic fish of claim **1** wherein said fish is a teleost fish.

8. The transgenic fish of claim **7** wherein said fish is selected from the group consisting of a medaka, a zebrafish, a mummichog, a killifish, a channel catfish, a common carp and a trout.

9. The transgenic fish of claim **1** wherein said fish is sterile.

10. The transgenic fish of claim **1** that is hemizygous for the mutation target nucleic acid sequence.

11. The transgenic fish of claim **1** that is homozygous for the mutation target nucleic acid sequence.

12. A method for detecting mutations in the DNA of a transgenic fish comprising:

providing a transgenic fish whose genomic DNA comprises a recoverable assayable mutation target nucleic acid sequence operably linked to a bacteriophage lambda-derived transgene construct;

recovering genomic DNA comprising the mutation target nucleic acid operably linked to the bacteriophage lambda-derived transgene construct;

transferring the genomic DNA into a bacterial host; and bioassaying in the bacterial host for the presence of a mutation in the mutation target nucleic acid sequence.

13. The method of claim **12** wherein the recovering step comprises extracting the genomic DNA from the fish to yield extracted genomic DNA comprising bacteriophage lambda-derived DNA comprising the assayable mutation target nucleic acid sequence and chromosomal DNA.

14. The method of claim **13** wherein the recovering step further comprises packaging the bacteriophage lambda-derived DNA to yield packaged phage.

15. The method of claim **14** wherein the transferring step comprises introducing the packaged phage into a bacterial host.

16. The method of claim **15** wherein the mutation target nucleic acid sequence comprises at least one nucleic acid sequence selected from the group consisting of the cII gene, the cII mRNA ribosome binding site and the cII protein-activated P_{RE} promoter sequence of bacteriophage lambda.

17. The method of claim **16** wherein the bacterial host comprises an hfl$^-$ *E. coli* strain.

18. The method of claim **15** wherein the mutation target nucleic acid sequence comprises at least one nucleic acid sequence selected from the group consisting of the lacI gene, the lacZ gene and the lac promoter sequence.

19. The method of claim **16** wherein the bacterial host comprises an *E. coli* strain that produces a polypeptide that complements the polypeptide encoded by the alacZ region of the lacZ gene to form active β-galactosidase.

20. The method of claim **12** wherein the providing step comprises providing a transgenic fish that has been or is suspected of having been exposed to a mutagen.

21. The method of claim **12** further comprising exposing the transgenic fish to a mutagen prior to recovering the DNA comprising the mutation target nucleic acid sequence.

22. The method of claim **21** wherein the mutagen is selected from the group consisting of a chemical, a radioisotope and electromagnetic radiation.

23. The method of claim **22** further comprising analyzing the mutation in the mutation target nucleic acid sequence.

24. The method of claim **23** wherein analyzing the mutation comprises determining the nucleic acid sequence of the mutation target nucleic acid sequence.

25. A method for evaluating the mutagenicity of a suspected mutagen comprising:

exposing a transgenic fish to a suspected mutagen, wherein the genomic DNA of the transgenic fish comprises a recoverable assayable mutation target nucleic acid sequence operably linked to a bacteriophage lambda-derived transgene construct;

recovering genomic DNA comprising the mutation target nucleic acid sequence operably linked to the bacteriophage lambda-derived transgene construct;

transferring the genomic DNA into a bacterial host; and bioassaying in the bacterial host for the presence of a mutation in the mutation target nucleic acid sequence.

26. The method of claim **25** wherein the mutation target nucleic acid sequence comprises at least one nucleic acid sequence selected from the group consisting of the lacI gene, the lacZ gene, the lac promoter sequence, the cII gene, the cII mRNA ribosome binding site and the cII protein-activated P_{RE} promoter sequence.

27. The method of claim **25** further comprising analyzing the mutation in the mutation target nucleic acid sequence.

28. The method of claim **27** wherein analyzing the mutation comprises determining the nucleic acid sequence of the mutation target nucleic acid sequence.

29. The method of claim **27** wherein analyzing the mutation comprises determining a mutation spectrum of the suspected mutagen.

30. A method for making a transgenic fish for mutagenesis detection, said method comprising microinjecting heterologous DNA into a one-cell fish embryo, and incubating the microinjected embryo such that the embryo develops into a fish, wherein the heterologous DNA comprises a recoverable assayable mutation target nucleic acid sequence operably linked to a bacteriophage lambda-derived transgene construct, the mutation target nucleic acid sequence being capable of being recovered, transferred into a bacterial host, and bioassayed in the bacterial host for the presence of a mutation.

31. The method of claim **30** wherein the mutation target nucleic acid sequence comprises at least one nucleic acid sequence selected from the group consisting of the lacI gene, the lacZ gene, the lac promoter sequence, the cII gene, the cII mRNA ribosome binding site and the cII protein-activated P_{RE} promoter sequence.

32. The method of claim **30** wherein the heterologous DNA is microinjected through the micropyle within about 5 minutes following fertilization.

33. A mutagenesis assay system comprising:

a transgenic fish whose genomic DNA comprises a recoverable assayable mutation target nucleic acid sequence operably linked to a bacteriophage lambda-derived transgene construct; and

a bacterial cell bioassay system for use in detecting the presence of a mutation in the target nucleic acid sequence that has been recovered from the transgenic fish after exposure to a potential mutagen, the bacterial cell bioassay system comprising a bacterial cell into which the target nucleic acid sequence has been introduced.

34. The mutagenesis assay system of claim **33** wherein the mutation target nucleic acid sequence comprises at least one nucleic acid sequence selected from the group consisting of the lacI gene, the lacZ gene, the lac promoter sequence, the cII gene, the cII mRNA ribosome binding site and the cII protein-activated P_{RE} promoter sequence.

35. The mutagenesis assay system of claim **33** wherein the transgenic fish is sterile.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 6,307,121 B1
DATED : October 23, 2001
INVENTOR(S) : Richard N. Winn

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,
Item [56], **References Cited**, OTHER PUBLICATIONS,
Amanuma et al. reference, please delete "Detectig" and insert -- Detecting -- therefor.
Black reference, please delete "Carcinogenesis" and insert -- Carcinogenic -- therefor.
Gallagher et al. reference, please delete "Demethylbenz" and insert -- Dimethylbenz -- therefor.
Schwarz et al. reference, please delete "o" and insert -- O -- therefor.
http://www.stratagene.com/vol110 3/figures/p100-101.htm reference, please delete "γLIZ" and insert -- λLIZ -- therefor.

Column 27,
Line 33, please delete "alacZ" and insert -- *alacZ* -- therefor.

Column 28,
Line 30, please delete "laci" and insert -- *lac*I -- therefor.

Signed and Sealed this

Third Day of September, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

EXHIBIT 3

US006472583B1

(12) **United States Patent**
Winn

(10) **Patent No.: US 6,472,583 B1**
(45) **Date of Patent: *Oct. 29, 2002**

(54) **PLASMID-BASED MUTATION DETECTION SYSTEM IN TRANSGENIC FISH**

(75) Inventor: **Richard N. Winn**, Athens, GA (US)

(73) Assignee: **The University of Georgia Research Foundation, Inc.**, Athens, GA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **09/427,218**

(22) Filed: **Oct. 26, 1999**

Related U.S. Application Data

(60) Provisional application No. 60/105,751, filed on Oct. 26, 1998.

(51) **Int. Cl.**[7] **G01N 33/00**; A01K 67/027; C12N 15/00

(52) **U.S. Cl.** **800/3**; 800/20; 800/21; 800/25

(58) **Field of Search** 800/20, 3, 21, 800/25

(56) **References Cited**

U.S. PATENT DOCUMENTS

5,347,075 A	9/1994	Sorge	800/2
5,470,706 A	11/1995	Vijg et al.	435/6
5,510,099 A	4/1996	Short et al.	424/9.2
5,545,808 A	8/1996	Hew et al.	800/2
5,589,155 A	12/1996	Sorge et al.	424/9.2
5,602,300 A	2/1997	Gossen et al.	800/2
5,817,290 A	10/1998	Vijg et al.	424/9.2
5,824,287 A	10/1998	Sorge et al.	424/9.2
6,114,600 A	9/2000	Ow et al.	800/278
6,307,121 B1 *	10/2001	Winn	

FOREIGN PATENT DOCUMENTS

EP	0 289 121 B1	11/1988
EP	353 812 B1	1/1994
JP	2618973	1/1989
JP	96 029 093 B2	5/1990
JP	08 205708	8/1996
NL	8801826	2/1990
NL	9100567	11/1992
WO	WO 91/15579	10/1991
WO	WO 92/17605	10/1992
WO	WO 93/15769	8/1993
WO	WO 93/17123	9/1993
WO	WO 97/05484	2/1997
WO	WO 99/62333	12/1999
WO	WO 00/24246	5/2000

OTHER PUBLICATIONS

Winn et. al. , Detection of mutations in transgenic fish carrying a bacteriophage transgene target, 2000; PNAS, vol. 97:12655–12660.*

Amanuma et al. Nature Genetics 18:62–65, 2000.*

Bayer et al., "A Transgene Containing lacZ is Expressed in Primary Sensory Neurons in Zebrafish," *Development*, 115:421–426 (1991).

Lin, et al., "lacZ Expression in Germline Transgenic Zebrafish Can Be Detected in Living Embryos," *Developmental Biology*, 161:77–83 (1994).

Article: "Scientists fish for toxicity clues," *Chemistry & Industry*, pp. 131 (1999).

Fosgate, Ed., "Fish may soon replace lab mice," *UGA, School of Forrest Resources, Alumini Association Publication, Forresters'* Log, pp. 6 and 8 (1999).

Bearzotti et al., "Gene expression following transfection of fish cells," *Journal of Biotechnology*, 26:318–325 (1992).

Detrick, "Transgenic Fish Model for Mutagenicity Testing," *Crisp Data Base National Institute of Health*, located at website: alt1.csa.com/htbin/ids51/procskel.cgi, Abstract, 1 page (1994).

Fosgate, "Transgenic fish may give lab mice day off," *Georgia Magazine*, p. 11 (1999).

Susman, "Gene–altered fish check new chemicals," located at website: news.chemicalonline.com/wires/19990124–421734772.htm, 2 pages. (1999).

Torassa, "Animals Hold Key to Cures," located at website: abcnews.go.coM:80/sections/science/DailyNews/scorpions9990125.htm, 2 pages. (1999).

"MutaPlax™ Transgenic Lambda DNA Packaging System," [online] [retrieved May 7, 1998]. Retrieved from the Internet: interscience.com/epicentre/mutplxc2.html, 1 page. 1998.

"MutaPlax cII–Select™ Packaging and Selection Kit," [online][retrieved May 28, 1998]. Retrieved from the Internet: interscience.com/epicentre/multiplxc2.html, 1 page. 1998.

"A short description of the lambda cII system," [online]. Biology Dept., University of Victoria, Canada, [retrieved on May 8, 1998]. Retrieved from the Internet: darwin.ceh.uvic.ca/bigblue/cci–info.htm, 1 page. 1998.

"A short description of the Big Blue system," [online]. Biology Dept., University of Victoria, Canada, [retrieved on May 28, 1998]. Retrieved from the Internet: <URL:http://darwin.ceh.uvic.ca/bigblue/bbinfo.htm>, 1 page. 1998.

"A short description of the lambda cII system," [online]. Biology Dept., University of Victoria, Canada, [retrieved on May 8, 1998]. Retrieved from the Internet: <URL:http://darwin.ceh.uvic.ca/bigblue/cci–info.htm>, 1 page. 1998.

(List continued on next page.)

Primary Examiner—Dave T. Nguyen
Assistant Examiner—Ram R. Shukla
(74) *Attorney, Agent, or Firm*—Mueting, Raasch & Gebhardt, P.A.

(57) **ABSTRACT**

The present invention provides transgenic fish whose somatic and germ cells contain a genomically integrated plasmid containing a heterologous mutation target nucleic acid sequence that is detectable via bioassay in a bacterial cell into which the target nucleic acid has been introduced. The frequency and character of mutations in the mutatable target nucleic acid sequence following exposure of the transgenic fish to one or more potentially mutagenic agents can thus be evaluated.

32 Claims, 1 Drawing Sheet

OTHER PUBLICATIONS

Barnes, "Fish Embryonal Stem Cells for Development of Transgenics," Grant No. 5 R01 ES 06011–06 [online] Oregon State University [retrieved Mar. 24, 1998]. Retrieved from the Internet: <URL:http://cos.gdb.org/cgi/bin/open/feddb/NIH/nih/81168666/81171512/data3/work/db–annex/nih/nih.data/seed=fish+transgenic+ak at cos.gdb.org>, 1 page. 1998.

"Big Blue® λLIZ Shuttle Vector," [online]. Strategene [retrieved May 28, 1998]. Retrieved from the Internet: <URL:http://www.stratagene.com/vol10__3/figures/p100–101–fl.htm>, 1 page. 1998.

Burkhart et al., "Non–Mammalian and Environmental Sentinels in Human Health: "Back to the Future?"," *Human and Ecological Risk Assessment*, 3(3):309–328 (1997).

Burkhart et al., "Spontaneous and Induced Mutagenesis in Transgenic Animals Containing ΦX174," Abstract, *Environmental and Molecular Mutagenesis*, 21(22):9 (1993).

Burkhart, "Comparative Mutagenesis in Aquatic Models, Rodents, and Cells in Culture," Abstract, Grant No. Z01 ES21187–02 [online]. NIH Intramural [retrieved Mar. 24, 1998]. Retrieved from the Internet: <URL:http://cos.gdb.org/cgi–bin/open/feddb/NIH/nih/183654826/183657932/data3/work/db–annex/nih/nih.data/seed=fish+transgenic+ak at cos.gdb.org>, 1 page. 1998.

Chen, "Transgenic Fish as a Model for Environmental Toxicology," Abstract, Grant No. 5 R01 RR11680–03 [online]. University of Connecticut STORRS [retrieved Mar. 24, 1998]. Retrieved from Internet <URL:http://cos.gdb.org/cgi–bin/open/feddb/NIH/nih/177554210/17558224/data3/work/db–annex/nih/nih.data/seed=fish+transgenic+ak at cos.gdb.org>, 1 page. 1998.

Dycaico et al., "The use of shuttle vectors for mutation analysis in transgenic mice and rats," *Mutation Research*, 307(2):461–478 (1994).

Epicentre Technologies, "cII Primer" Product Information, Catalog #P67PL1, and #P67PL2, 1 page (1996).

Gallagher et al., "A Comparative Approach to 7,12–Dimethylbenz[A]Anthracene Effects: Metabolism and Mutagenesis in Mice and Fish," Abstract, *Environment and Molecular Mutagenesis*, 29(28):16 (1997).

Grunwald, "Embryo Stem Cell Cultures and Gene Transfer in Zebrafish," Abstract, Grant No. 5 R01 RR10253–04 [online]. University of Utah [retrieved Mar. 24, 1998]. Retrieved fom the Internet: <URL:http://cos.gdb.org/cgi–bin/open/feddb/NIH/nih/176701197/176704983/data3/work/db–annex/nih/nih.data/seed=fish+transgenic+ak at cos.gdb.org>, 1 page. 1998.

Gu et al., "Development of λ–based complementation assay for the preliminary localization of lacI mutants from the Big Blue™ mouse: implications for a DNA–sequencing strategy," *Mutation Research*, 307(2):533–540 (1994).

Guyomard et al.; "Integration and germ line transmission of foreign genes microinjected into fertilized trout eggs," *Biochimie*, 71(7):857–863 (1989).

Houdebine et al., "Transgenesis in fish," *Experientia*, 47(9);891–897 (1991).

International Agency for Research on Cancer (IARC), IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Humans, 7, IARC, Lyon., 253–260 (1974).

Kaiser et al., "Fishing for Toxic Chemicals," *Science*, 283(5403):775, 777 (Feb. 5, 1999).

Kohler et al., "Spectra of spontaneous and mutagen–induced mutations in the lacI gene in transgenic mice," *Proc. Natl. Acad. Sci. USA*, 88(18):7958–7962 (1991).

"λSelect–cII™Mutation Detection Sytem for Big Blue® Rodents," Instruction Manual, Stratagene, Catalog #720120, Revision #028001, pp. 1–21 (1998).

Liu et al., "Development of Expression Vectors for Transgenic Fish," *Biotechnology*, 8(12):1268–1272 (1990).

Lubzens et al., "Cryopreservation as a Tool in Aquaculture," Abstract only, *Israeli Journal of Aquaculture*, 44(4):137 (1992).

Murray et al., eds., *Transgenic Animals in Agriculture*, CABI Publishing, New York, Title page, publication page, and pp. 33, 88–89, 91, 93–95, 217–218, and 224–229 (1999).

"MutaPlax™ Transgenic Lambda DNA Packaging System." [online][retrieved May 7, 1998]. Retrieved from the Internet: <URL:http://www.interscience.com/epicentre/mutplxc2.html>, 1 page. 1998.

"MutaPlax cII–Select™ Packaging and Selection Kit," [online][retrieved May 28, 1998]. Retrieved from the Internet: <URL:http://www.interscience.com/epicentre/mutplxc2.html>, 1 page. 1998.

Nebert, "Transgenic Zebrafish–Sentinel for Aquatic Pollution", Abstract, Grant No. 5 R01 ES07058–02 [online]. University of Cincinnati [retrieved Mar. 24, 1998]. Retrieved from the Internet: <URL:http://cos.gdb.org.cgi–bin/open/feddb/NIH/nih/81969991/81973169/data3/work/db–annex/nih/nih.data/seed=fish+transgenic+ak at cos.gdb.org>, 1 page. 1998.

Provost et al., "Transgenic systems for in vivo mutation analysis," *Mutation Research*, 288(1):133–149 (1993).

Rokkones et al., "Microinjection and expression of a mouse metallothionein human growth hormone fusion gene in fertilized salmonid eggs," *Journal of Comparative Physiology B*, 158(6):751–758 (1989).

Sambrook et al., "Preparation of Reagents and Buffers Used in Molecular Cloning," *Molecular Cloning* 2nd Ed., Cold Spring Harbor Press, p.B.13 (1989).

Schwarz et al., "Nucleotide sequence of cro, cII and part of the O gene in phage λ DNA," *Nature*, 272(5652):410–414 (1978).

Shimada et al., "Some Characteristics of Radiation–or Enu–induced Mutations at the b Locus of the Medaka, Oryzias Latipes," Abstract GE 3, *Physiology, Genetics*, 7(6):1053 (1990).

Shimada et al., "Further Characterization of Radiation–or Enu–induced Mutations at the b Locus of the Medaka, *Oryzias Latipes*," Abstract, *Genetics* 8(6):1127 (1991).

Skopek et al., "Relative Sensitivity of the Endogenous hprt Gene and lacI Transgene in ENU–Treated Big Blue™ B6C3F1 Mice," *Environmental and Molecular Mutagenesis*, 26(1):9–15 (1995).

Strategene, *Big Blue™ Transgenic Mouse Mutagenesis Assay System*, Instruction Manual, La Jolla, CA, Title Page and table of contents only, 2 pages. (1992).

Strategene Online, "Technical Information—Frequently Asked Questions—Big Blue®," [online]. Strategene [retrieved Mar. 24, 1998]. Retrieved from the Internet: <URL:http://www.stratagene.com/tech__ref/big__blue/faq.htm>, 3 pages. 1998.

Strategene Online, "Strategies Newsletters—A Positive Selection Assay for Mutation Analysis in Big Blue® Animals," [online]. Strategene [retrieved May 28, 1998]. Retrieved from the Internet: <URL:http://www.stratagene.com/vol10_3/p100–101.html>, 2 pages. 1998.

Winn, "Transgenic Fish Model for Mutation Detection," Grant No. 1 R24 RR11733–01A1 [online]. University of Georgia [retrieved Mar. 24, 1998]. Retrieved from the Internet: <URL:http://cos.gdb.org/cgi–bin/open/feddb/NIH/nih/177576697/177580360/data3/work/db–annex/nih/nih-.data/seed=fish+lambda+ak at cos.gdb.org 1 page. 1998.

Bailey et al., "Initiation, Promotion, and Inhibition of Carcinogenesis in Rainbow Trout," *Environ. Health Perspect.*, 71:147–153 (1987).

Black, "Aquatic Animal Neoplasia as an Indicator for Carcinogenic Hazards to Man," *Hazard Assessment of Chemicals: Current Developments*, vol. 3, Saxena, ed., Academic Press, New York, Title page, publication page, table of contents and pp. 181–232 (1984).

Boerrigter et al., "Plasmid–based transgenic mouse model for studying in vivo mutations," *Nature*, 377(6550):657–659 (1995).

Boerrigter et al., "Sources of Variability in Mutant Frequency Determinations in Different Organs of lacZ Plasmid–based Transgenic Mice: Experimental Features and Statistical Analysis," *Env. Mol. Mutagenesis*, 29(3):221–229 (1997).

Boerrigter et al., "High Sensitivity for Color Mutants in lacZ Plasmid–Based Transgenic Mice, as Detected by Positive Selection," *Env. Mol. Mutagenesis*, 32(2):148–154 (1998).

Burkhart et al., ENU–induced mutagenesis at a single A:T base pair in transgenic mice containing ΦX174 *Mutation Res.*, 292:69–81 (1993).

Culp et al., "High–frequency germ–line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs," *Proc. Natl. Acad. Sci. USA*, 88(18):7953–7957 (1991).

Doll'' et al., "Evaluation of a plasmid–based transgenic mouse model for detecting in vivo mutations," *Mutagenesis*, 11(1):111–118 (1996).

Douglas et al., "Sequence spectra of spontaneous lacZ gene mutations in transgenic mouse somatic and germline tissues", *Mutagenesis*, 9(5):451–458 (1994).

Driever et al., "Zebrafish: genetic tools for studying vertebrate development," *Trends Genet.*, 10(5):152–159 (1994).

Driever et al., "A genetic screen for mutations affecting embryogenesis in zebrafish," *Development*, 123:37–46 (1996).

DuBridge et al., "Recombinant shuttle vectors for the study of mutation in mammalian cells," *Mutagenesis*, 3(1):1–9 (1988).

Dycaico et al., "The use of shuttle vectors for mutation analysis in transgenic mice and rats," *Mutation Res.*, 307:461–478 (1994).

Goldsworthy et al., "Transgenic Animals in Toxicology," *Fund Appl. Toxicol.*, 22:8–19 (1994).

Gossen et al., "Efficient rescue of integrated shuttle vectors from transgenic mice: A model for studying mutations in vivo," *Proc. Natl. Acad. Sci.*, 86:7971–7975 (1989).

Gossen et al., "Plasmid Rescue form Transgenic Mouse DNA Using LacI Repressor Protein Conjugated to Magnetic Beads," *BioTechniques*, 14(4):624–629 (1993).

Gossen et al., "Spontaneous and X–ray induced deletion mutations in a LacZ plasmid–based transgenic mouse model," *Mut. Res.*, 331:89–97 (1995).

Hawkins et al., "Chapter 14: Carcinogenicity Tests Using Aquarium Fish," *Fundamentals of Aquatic Toxicology: Effects, Environmental Rate, and Risk Assessment*, Rand, ed., Taylor and Francis, pp. 421–446 (1995).

Hendricks, "Chemical Carcinogenesis in Fish," *Aquatic Toxicology*, Weber, ed., Raven Press, NY, pp. 149–211 (1982).

Hinton et al., "Cytological changes during progression of neoplasia in selected fish species," *Aquat. Toxicol.*, 11:77–112 (1988).

Inoue et al., "Stage–dependent expression of the chicken δ–crystallin gene in transgenic fish embryos," *Cell Differ. Dev.*, 27:57–68 (1989).

Inoue et al., "Electroporation as a new technique for producing transgenic fish," *Cell Differ. Dev.*, 29(2):123–128 (1990).

International Agency for Research on Cancer (IARC), World Health Organization, "Some anti–thyroid and related substances, nitrofurans and industrial chemicals," *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Man*, meeting of the IARC working groups, Lyon, Feb. 4–11 and Jun. 18–24, 1974, pp. 253–260 (1974).

Ishikawa et al., "Importance of Hepatic Neoplasms in Lower Vertebrate Animals as a Tool in Cancer Research," *J. Toxicol. Environ. Health*, 5(2–3):537–550 (1979).

Ishikawa et al., "Usefulness of the Medaka, *Oryzias latipes*, as a Test Apimal: DNA Repair Processes in Medaka Exposed to Carcinogens," *Use of Small Fish Species in Carcinogenicity Testing, Natl. Cancer Inst. Monograph 65*, U.S. Dept. of Health and Human Services, National Institutes of Health, pp. 35–43 (1984).

Jakubczak et al., "Analysis of genetic instability during mammary tumor progression using a novel selection–based assay for in vivo mutations in a bacteriophage λ transgene target," *Proc. Natl. Acad. Sci. USA*, 93(17):9073–9078 (1996).

Lebkowski et al., "The lacI shuttle: Rapid analysis of the mutagenic specificity of ultraviolet light in human cells," *Proc. Natl. Acad. Sci. USA*, 82:8606–8610 (1985).

Lewis et al., "The Nature of Spontaneous and Induced Electrophoretically Detected Mutations in the Mouse," *Prog. Clin. Biol. Res.*, 209B:359–365 (1986).

Lu et al., "Integration: expression, and germ–like transmission of foreign growth hormone genes in medaka (*Oryzias latipes*)," *Mol. Mar. Biol. Biotechnol.*, 1(½):366–375 (1992).

Lu et al., "Pantropic retroviral vector integration, expression, and germline transmission in medaka (*Oryzias latipes*)," *Mol. Mar. Biol. Biotechnol.*, 6(4):289–295 (1997).

Malling et al. ,"Use of ΦX 174 as a shuttle vector for the study of in vivo mammalian mutagenesis," *Mutation Res.*, 212:11–21 (1989).

Masahito et al., "Fish Tumors and their Importance in Cancer Research," *Jpn. J. Cancer Res.*, 79(5):545–555 (1988).

Metcalfe, "Tests for Predicting Carcinogenicity in Fish," *CRC Rev. Aquat. Sci.*, 1(1):111–129 (1989).

Mirsalis et al., "Induction of hepatic mutations in lacI transgenic mice," *Mutagenesis*, 8:265–271 (1993).

Mirsalis et al., "Transgenic Animal Models for Detection of In Vivo Mutations," *Ann. Rev. Pharmacol. Toxicol.*, 35:145–164 (1995).

Müller et al., "Introducing foreign genes into fish eggs with electroporated sperm as a carrier," *Mol. Mar. Biol. Biotechnol.*, 1(4/5):276–281 (1992).

Müller et al., "Efficient transient expression system based on square pulse electroporation and in vivo luciferase assay of fertilized fish eggs," *FEBS Lett.*, 324(1):27–32 (1993).

Murakami et al., "Micromachined electroporation system for transgenic fish," *J. Biotechnol.*, 34:35–42 (1994).

Ozato et al., "Production of transgenic fish: introduction and expression of chicken δ–crystallin gene in medaka embryos," *Cell Differ.*, 19:237–244 (1986).

Postlethwait et al., "Zebrafish genomics: from mutants to genes," *Trends Genet.*, 13(5):183–190 (1997).

Powers, "Fish as Model Systems," *Science*, 246:352–358 (1989).

Powers et al., "Electroporation: a method for transferring genes into the gametes of zebrafish (*Brachydanio rerio*), channel catfish (*Ictalurus punctatus*), and common carp (*Cyprinus carpio*)," *Mol. Mar. Biol. Biotechnol.*, 1(4/5):301–308 (1992).

Riley et al., "Efficient induction of point mutations allowing recovery of specific locus mutations in zebrafish," *Proc. Natl. Acad. Sci. USA*, 92(13):5997–6001 (1995).

Rogers et al., "Intralaboratory optimization and standardization of mutant screening conditions used for a lambda/lacI transgenic mouse mutagenesis assay (I)," *Mutation Res.*, 32:57–66 (1995).

Russell et al., "The Mouse Specific–Locus Test with Agents Other Than Radiations: Interpretation of Data and Recommendations for Future Work," *Mutation Res.*, 86:329–354 (1981).

Rüther et al., "Easy identification of cDNA clones," *EMBO J.*, 2(10):1791–1794 (1983).

Shimada et al., "Some Characteristics of Radiation–or ENU-Induced Mutations at the b Locus of the Medaka, *Oryzias latipes*," Abstract GE 3 *Zoological Sci.* (Tokyo), 7(6):1053 (1990).

Shimada et al., "Further Characterization of Radiation–or ENU–Induced Mutations at the b Locus of the Medaka, *Oryzias latipes*," Abstract, *Zoological Sci.* (Tokyo), 8(6):1127 (1991).

Singer, "All oxygens in nucleic acids react with carcinogenic ethylating agents," *Nature*, 264:333–339 (1976).

Singer et al., "Oxygens in DNA are main targets for ethylnitrosourea in normal and xeroderma pigmentosum fibroblasts and fetal rat brain cells," *Nature*, 276:85–88 (1978).

Solnica–Krezel et al., "Efficient Recovery of ENU–Induced Mutations From the Zebrafish Germline," *Genetics*, 136:1401–1420 (1994).

Streisinger et al., "Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*)," *Nature*, 291:293–296 (1981).

Stuart et al., "Replication, integration and stable germ–like transmission of foreign sequences injected into early zebrafish embryos," *Development*, 103(2):403–412 (1988).

Symonds et al., "Development of a mass gene transfer method in chinook salmon: optimization of gene transfer by electroporated sperm," *Mol. Mar. Biol. Biotechnol.*, 3(2):104–111 (1994).

Szelei et al., "Entrapment of high–molecular–mass DNA molecules in liposomes for the genetic transformation of animal cells," *Biochem. J.*, 259:549–553 (1989).

Tamiya et al., "Spatial imaging of luciferase gene expression in transgenic fish," *Nucleic Acids Res.*, 18(4):1072 (1990).

Tao et al., "Comparison of somatic mutation in a transgenic versus host locus," *Proc. Natl. Acad. Sci., USA* 90(22):10681–10685 (1993).

Tennant et al., "Prediction of Chemical Carcinogenicity in Rodents from in Vitro Genetic Toxicity Assays," *Science*, 236:933–941 (1987).

Tsai et al., "Initiation of the transgenic lacZ gene expression in medaka (*Oryzias latipes*) embryos," *Mol. Mar. Biol. Biotechnol.*, 4(1):1–9 (1995).

Valcovic et al., "An Approach to Measuring Germinal Mutations in the Mouse," *Environ. Health Perspect.*, 6:201–205 (1973).

Van Beneden et al., "Oncogenes in Hematopoietic and Hepatic Fish Neoplasms," *Cancer Res.* (Suppl.), 50:5671s–5674s (1990).

Vijg et al., "Ch 29:Bacteriophage Lambda and Plasmid lacZ Transgenic Mice for Studying Mutations in Vivo," *Technologies for Detection of DNA Damage and Mutations*, Plenum Press, NY, Title page, publication page, table of contents and pp. 391–410 (1996).

Vogelstein et al., "Genetic Alterations During Colorectal-Tumor Development," *N. Engl. J. Med.*, 319(9):525–532 (1988).

Walker et al., "A Small Fish Model for Assessing Cancer Risk at Low Carcinogen Concentrations," Abstract 302, Abstracts for the 31st Annual Meeting of the Society of Toxicology, Feb., 1992, *The Toxicology*, 12(1):97 (1992).

Winn et al., "Transfer, Methylation and Spontaneous Mutation Frequency of ΦX174am3cs70 sequences in Medaka (*Oryzias latipes*) and Mummichog (*Fundulus heteroclitus*): Implications for Gene Transfer and Environmental Mutagenesis in Aquatic Species," *Marine Environ. Res.*, 40(3):247–265 (1995).

Yamamoto, *Series of Stock Culture in Biological Field: Medaka (killifish): Biology and Strains* 1st *Ed.*, Keigaku Publishing Co., Tokyo, Title page, publication page and table of contents only, 3 pages. (1975).

Zelenin et al., "The delivery of foreign genes into fertilized fish eggs using high–velocity microprojectiles," *FEBS Lett.*, 287(1–2):118–120 (1991).

* cited by examiner

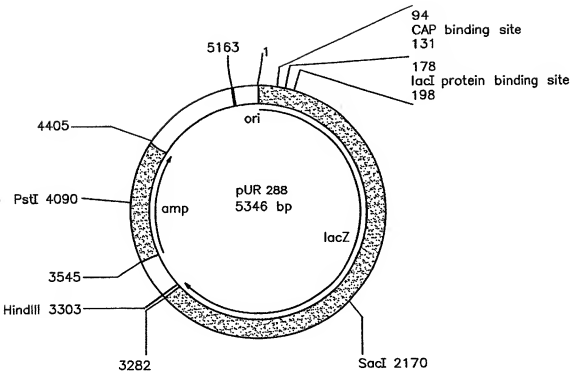

94
CAP binding site
131
178
lacI protein binding site
198
5163
1
ori
4405
pUR 288
5346 bp
PstI 4090
amp
lacZ
3545
HindIII 3303
3282
SacI 2170

FIG. 1

PLASMID-BASED MUTATION DETECTION SYSTEM IN TRANSGENIC FISH

This application claims the benefit of U.S. Provisional Application Ser. No. 60/105,751, filed Oct. 26, 1998.

FIELD OF THE INVENTION

This invention relates to a transgenic fish carrying a plasmid-based marker, and in particular relates to a transgenic fish for use in evaluating the effect of a potential mutagen. The transgenic fish is exposed to the mutagen, and mutagenesis is detected by assaying for a mutation target nucleic acid sequence present as a genomically integrated transgene in the transgenic fish.

BACKGROUND OF THE INVENTION

The health risk posed by exposure to mutagenic agents in the environment remains an important concern as it is known that induction of mutations may lead to various somatic or inherited diseases. In particular, cancer has been shown to result from a series of mutations in specific oncogenes and tumor suppressor genes (Vogelstein et al., *N. Engl. J. Med.* 319: 525–532 (1988)). Despite the recognition of the role of induced mutation as an important event leading to disease, there are few methods available for the assessment of genetic hazard, or focus on the study of gene mutations as they occur at the DNA level in vivo. As a result, there is an immediate need to develop sensitive and biologically relevant methods that can be applied to the study of the mechanisms of mutagenesis and hazard assessment.

There are two practical requirements common to any study of mutagenesis: 1) the specific loci to be examined should be sensitive to mutation induction, and 2) the mutants must be recovered in sufficient numbers. Until recently, progress in the analysis of gene mutations directly at the DNA level was limited by the standard molecular techniques and the available endogenous genes. During past volts, the most relevant assays for induction of transmissible mutations have been based on the appearance of visible or biochemical mutations among the offspring of exposed mice (L. B. Russell et al., *Mutation Res.* 86: 329–354 (1981); L. R. Valsovic et al., *Environ. Health Perspect.* 6:201–205 (1973); S. E. Lewis et al., *Prog. Clin. Biol. Res.* 209B. 359–365 1986)). These tests cannot be practically applied to large numbers of compounds because they require extensive resources and very large numbers of animals. The tests also fail to provide information regarding somatic mutagenesis or clustering of mutations, which may be important in the understanding of the development of various diseases.

In order to circumvent some of the problems inherent in rodent assays, short-term mutagenicity tests were developed, based on the assumption that many of the chemicals toxic to rodents would also be genotoxic to bacteria. However, an analysis by the National Toxicology Program (R. W. Tennant et al., *Science* 236:933–941 (1987)) revealed significant differences in results between rodent and bacterial tests. This failure of predictive correlation may be related to: 1) a lack of understanding of the roles mutation plays in cell transformation, and 2) differences between animals and bacterial cells in terms of exposure, biological milieu, metabolism, replication and repair. While comparisons between animals and animal cells in culture provide appropriate genomic similarity, there are few known biological markers for mutation of cells in culture. The biological markers that have been identified are restricted to specific cell types and therefore are of limited use for in vivo comparisons.

There thus remains a need to combine the simplicity of short-term in vitro assays with in vivo studies. Ultimately, reliable and realistic hazard assessment and informative mechanistic studies of mutagenesis require the development of practical methods for evaluating somatic and genetic events in whole animals exposed to environmental agents. New approaches that use recombinant DNA and gene transfer techniques to develop transgenic animal models offer significant promise for in vivo studies of mutagenesis, cancer, birth defects and other diseases (T. L. Goldsworthy et al., *Fund. Appl. Toxicol.* 22:8–19 (1994)). Transgenic rodents that carry genes specifically designed for the quantitation of spontaneous and induced mutations are currently available and represent a major advance in the study of mutagenesis by allowing rapid analysis of tissue-specific mutations in a whole organism following mutagenic agent exposure (J. C. Mirsalis et al., *Ann. Rev. Pharmacol. Toxicol.* 35:145–164 1995)).

To be effective, the transgenic approach as applied to mutagenesis should include the following components: 1) unique genes with known sequences; 2) a capacity to observe changes at the single gene copy level; 3) an easily attainable sample population of sufficient size to allow measurement of low frequency events; and 4) the ability to determine the exact nature of the mutation, independent of the host phenotype. Transgenic mutagenesis assay systems based on this approach rely on bacteriophage or plasmid shuttle vectors to carry a mutation target. The basic principle in this approach is that a recombinant gene which carries a mutation target (shuttle vector) is introduced into a host genome. Following exposure to a mutagen, the target gene is recovered to serve as an indicator of mutagenesis (reviewed by R B. Dubridge et al., *Mutagenesis* 3(1):1–9 (1988)).

Shuttle vectors currently in use include both bacteriophage-based and plasmid-based vectors. For example, the lambda (λ) bacteriophage-based recombinant vector combines cos site packaging for recovery of the phage sequence from the host DNA and uses the lacI, lacZ or cII genes as the target gene (J. S. Lebkowski et al., *Proc. Natl. Acad. Sci.* 82:8606–8610 (1985); J. A. Gossen et al., *Proc. Natl. Acad. Sci.* 86:7971–7975 (1989); J. L. Jakubczak et al., *Proc. Natl. Acad. Sci. U.S.A.*, 93:9073–9078 (1996)). Another system is based on the pUR288 plasmid vector which contains the lacZ sequence as the mutation target (M. Boerrigter et al., *Nature* 377:657–659 (1995); M. Dollé et al., *Mutagenesis* 11:111–118 (1996)). In both the λ and plasmid-based assays, mutation-induced inactivation of the lac genes are then detected histologically in *E. coli*. Another system is based on the bacteriophage φX174 integrated shuttle vector in which the vector is recovered by transfection. This vector is recovered from the transgenic host, transfected into a suitable *E. coli* host, and mutations at specific locations in the phage sequence are identified by suppressor-mediated selection on permissive and non-permissive *E. coli* (H. V. Malling et al., *Mutation Res.* 212:11–21 (1989); R. N. Winn et al., *Marine Environ. Res.* 40(3):247–265 (1995)).

A fundamental limitation of the bacteriophage-based mutation detection systems is their apparent inability to detect large-scale DNA deletions characteristically induced by clastogenic agents such as ionizing radiation (K. Tao et al., *Proc. Nat'l Acad. Sci, U.S.A.*, 90:10681–10685 (1993)). Most deletions reported thus far in the abased systems have only been 1–23 base pairs in length. Deletions in the range of hundreds of base pairs are rarely reported using bacteriophage-based mutagenesis detection assays (G. Dou-

glas et al., *Mutagenesis* 9:451–458 (1994)). Current estimates, depending upon the particular test system, are that up to 90% of radiation-induced mutations are thought to be DNA deletions. The bacteriophage shuttle-vector systems seem to have an inherent bias against detecting certain types of deletions primarily due to restrictive packaging and recovery requirements. It is speculated that since two intact cos-sites are required for the packaging of a single λ vector, any deletions that extend into regions adjacent to a transgene concatamer may prevent vector recovery.

A plasmid-based process for detecting mutations in whole animals is described in Gossen et al. (U.S. Pat. No. 5,602,300), but is limited to use in transgenic mammals, namely rodents. The plasmid pUR288, which contains a pBR322 Ori for replication, the ampicillin gene, and the whole lacZ gene including the lacZ operator sequence, was inserted into a bacteriophage lambda vector and transferred to the germ line of a mouse by means of microinjection of fertilized egg cells. The lacz-containing plasmid was purified from chromosomal DNA of a resulting transgenic mouse by treating the genomic DNA with a restriction enzyme (also known as a restriction endonuclease), then contacting the restriction digest to a solid support comprising LacI repressor protein (i.e., a lacZ operator binding material) to bind and isolate the plasmid. Gossen et al. (*Mut. Res.* 331:89–97 (1995)) also disclose incorporation of the linearized form of the plasmid directly into mammalian DNA (without using a bacteriophage vector); and detection of mutations in the lacZ gene by plasmid rescue as well as bacteriophage rescue. Vijg et al. (U.S. Pat. No. 5,817,290) teach a similar plasmid-based method for detection of mutations, except that the plasmid vector is recovered by simultaneously excising it from genomic DNA and contacting it with the solid affinity suppport.

There remains a need for an improved mutagenesis assay detection system capable of detecting mutagenic events that may be missed by bacteriophage-based systems. Additionally, alternative animal models are needed to extend and improve methods used to assess the potential genetic health risks posed by exposure to mutagens the environment.

SUMMARY OF THE INVENTION

A transgenic fish has been developed for use in a plasmid-based mutagenesis detection system. The system allows in vivo quantitation of By spontaneous and induced mutations using a recoverable mutation target nucleic acid sequence and assay system. After exposure of the transgenic fish to a mutagen, DNA is extracted from the fish tissue, and the mutation target nucleic acid sequence is recovered and analyzed for mutagenesis, preferably using a bioassay in indicator bacteria. In a preferred embodiment, the mutagenesis detection system of the invention is based on the pUR288 plasmid.

The transgenic fish of the invention is one whose somatic and germ cells contain at least one genomically integrated copy of a plasmid carrying an assayable mutation target nucleic acid sequence. The plasmid is preferably one that is suitable for cloning into *E. coli*. The invention further provides a transgenic fish gamete, including an transgenic fish egg or sperm cell, a transgenic fish embryo, and any other type of transgenic fish cell or cluster of cells, whether haploid, diploid, triploid or other zygosity having at least one genomically integrated copy of a plasmid comprising a mutation target nucleic acid sequence. As used herein, the term "embryo" includes a single cell fertilized egg (i.e., a zygote) as well as a multicellular developmental stage of the organism. Preferably, the plasmid is integrated into the fish's somatic and germ cells such that it is stable and inheritable. The transgenic fish or fish cell preferably contains a multiplicity of genomically integrated copies of the plasmid; more preferably, the multiple copies of the plasmid are integrated into the host organism's genome in a contiguous, head-to-tail orientation. Progeny of the transgenic fish containing at least one genomically integrated copy of the plasmid, and transgenic fish derived from a transgenic fish egg, sperm, embryo or other fish cell of the invention, are also included in the invention. A fish is "derived from" a transgenic fish egg, sperm cell, embryo or other cell if the transgenic fish egg, sperm cell, embryo or other cell contributes DNA to the fish's genomic DNA. For example, a transgenic embryo of the invention can develop into a transgenic fish of the invention; a transgenic egg of the invention can be fertilized to create a transgenic embryo of the invention that develops into a transgenic fish of the invention; a transgenic sperm cell of the invention can be used to fertilize an egg to create a transgenic embryo of the invention that develops into a transgenic fish of the invention; and a transgenic cell of the invention can be used to clone a transgenic fish of the invention. In some preferred embodiments of the invention, the transgenic fish is sterile. The present invention further includes a cell line derived from a transgenic fish embryo or other transgenic fish cell of the invention, which contains at least one copy of a plasmid carrying an assayable mutation target nucleic acid sequence.

The mutation target nucleic acid sequence is preferably one having a characteristic or function, or encoding a gene product having a characteristic or function, that is detectably altered when mutated, thereby allowing the nonmutated form of the nucleic acid sequence to be distinguished from the mutated form. In a particularly preferred embodiment, a mutation in the mutation target nucleic acid sequence is detectable via bioassay in a bacterial cell, such as an *E. coli* cell, into which a mutation target nucleic acid sequence that has been isolated from the fish or fish cell has been introduced. In this regard, a transgenic fish having a triploid genome is desirable because triploidy allows larger amount of DNA to be recovered. An increase in the amount of DNA recovered has many advantages. For example, it allows for more efficient detection of the mutation target nucleic acid. Moreover, fish having a triploid genome are typically sterile, which may be desirable for certain applications or studies. The assayable mutation target nucleic acid sequence is typically heterologous with respect to the fish genome. Preferably, the plasmid is integrated into the host organism's genome in a manner that avoids causing a detectable mutation in an endogenous gene of the host, thereby avoiding undesirably high background levels of mutation and reducing the sensitivity of the assay. The use of a smaller vector is preferred because the small size reduces the likelihood of physical disruption of one of the host cell's genes. Preferred mutation target nucleic acid sequences include the lacI gene, the lacZ gene, the lac promoter sequence, and the rpsL gene. Preferably, the lacZ gene includes the lacZ promoter.

In another embodiment, the invention includes a genomically identical population of transgenic fish, each of whose somatic and germ cells contain at least one genomically integrated copy of a plasmid comprising an assayable mutation target nucleic acid sequence. The genomically identical population is a unisex population and can be male or female. Preferred embodiments of the genomically identical transgenic fish population are essentially as described for the transgenic fish of the invention. In an alternative

embodiment, the invention includes a population of transgenic fish, i.e., an in-bred line, the members of which are not necessarily genomically identical but are homozygous with respect to genomically integrated plasmid.

Also provided is a method for mutation detection utilizing the transgenic fish or fish cell of the invention. This method is useful in evaluating the mutagenicity of various potential mutagens, such as chemical compounds, radioisotope emissions, and electromagnetic radiation. Mutations are detected in a mutation target nucleic acid sequence of a plasmid, wherein at least one copy of the plasmid has been integrated into the genomic DNA of the fish or fish cell. DNA containing the mutation target nucleic acid sequence is first recovered from the transgenic fish or fish cell, preferably by extracting the fish or fish cell DNA from the fish or fish cell, then cleaving the extracted DNA with a restriction endonuclease to yield at least one DNA fragment comprising the mutation target nucleic acid sequence derived from the plasmid, and multiple DNA fragments comprising chromosomal DNA. The DNA fragment that includes the mutation target nucleic acid sequence preferably includes substantially the entire plasmid, although it can contain a portion of the plasmid DNA, as long as it contains the mutation target nucleic acid sequence. Optionally, the method of mutation detection farther comprises separating the DNA fragment comprising the mutation target nucleic acid sequence from the multiple chromosomal fragments to isolate the mutation target DNA, although it should be understood. flat separation of the cleaved fragments comprising the mutation target DNA from the remaining chromosomal DNA is not required by the present method. If separation of the fragments is performed, the cleavage and the separation steps can be performed sequentially, or they can be performed simultaneously. In a particularly preferred embodiment of the method of the invention, wherein the mutation target nucleic acid sequence contains the lacZ gene, cleaved DNA fragments are separated by contacting the fragments with an affinity support comprising a lacZ operator binding material so as to immobilize the DNA fragment containing the mutation target nucleic acid sequence. After washing away the unbound DNA fragments, the bound DNA fragment is eluted from the support. The method further includes detection of the presence of a mutation in the mutation target nucleic acid sequence. Where the mutation target nucleic acid sequence contains the lacZ gene, mutations in the gene are preferably detected by transforming a host restriction-negative, lacZ^{-}, galE^{-} bacterial host with cleaved DNA comprising the mutation target nucleic acid sequence (whether or not the DNA fragments containing chromosomal DNA have been separated out); culturing the transformed bacteria on a lactose-containing or lactose analogue-containing medium; and selectively detecting a bacterial host that contains a mutation in the lacZ gene. Growth of the bacterial host is indicative of the existence of a mutation in the lacZ gene. Optionally, prior to transforming the bacterial host, the DNA comprising the mutation target nucleic acid test region is ligated to yield a circular DNA that is more efficiently electroporated. Also optionally, mutations in the mutation target nucleic acid sequence can be further analyzed, for example by nucleic acid sequence determination. When used to evaluate the mutagenicity of a particular agent, condition or event, the method further comprises, prior to extracting the fish DNA, exposing the transgenic fish or fish cell to the suspected mutagen.

The invention further includes a method for evaluating the mutagenicity of a suspected mutagen. A transgenic fish or fish cell of the invention is exposed to a suspected mutagen; the DNA containing the mutation target nucleic acid sequence is recovered from the transgenic fish or fish cell; and the presence of a mutation in the mutation target nucleic acid sequence is detected. Optionally, the mutated target nucleic acid sequence can be analyzed, for example by nucleic acid sequencing and the constructing a mutation spectrum.

Also included in the invention is a method for making a transgenic fish. Heterologous DNA is injected into a one-cell fish embryo, preferably through the micropyle, within about 10 minutes following fertilization, preferably within about 5 minutes following fertilization. A method for making a transgenic fish for mutagenesis detection includes microinjecting heterologous DNA into a one-cell fish embryo, wherein the heterologous DNA comprises a mutation target nucleic acid sequence, such as a lacZ gene.

The invention further includes a kit for detecting mutagenesis in transgenic fish comprising a genomically integrated plasmid comprising a mutation target nucleic acid. The kit includes, separately packaged, a host restriction-negative bacterial strain and a solid support that includes binding material capable of binding at least a portion of the plasmid. When used to detect mutagenesis in a transgenic fish having the lacZ gene as its mutation target nucleic acid sequence, the bacterial strain is preferably a host restriction-negative, lacZ^{-}, galE^{-} *E. coli* strain, and the solid support preferably includes a lac operator binding material such as β-galactosidase/LacI repressor fusion protein. The kit can optionally contain one or more additional components, such as a binding buffer to promote binding of the plasmid to the solid support, one or more restriction enzymes to excise the plasmid from the genomic DNA of the transgenic fish, an excision buffer, and a ligation for circularization of the excised plasmid prior to introduction of the plasmid into the bacterial host. The ligation buffer preferably contains a ligase, such as T4 ligase. The binding buffer and the excision buffer can, but need not be, the same buffer, so as to allow simultaneous excision of the plasmid and binding of the plasmid to the solid support.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of plasmid pUR288 (U.

Rüther et al., EMBO J. 2(10):1791–1794 (1983); J. Gossen et al., *Mut. Res.* 331:89–97 (1995); Gossen et al. (U.S. Pat. No. 5,602,300)).

DETAILED DESCRIPTION

The present invention provides a transgenic fish and fish cell, along with methods for detecting mutations in the DNA of a transgenic fish or a transgenic fish cell. The DNA of the transgenic fish of the invention contains one or more copies of a linearized plasmid containing a mutation target nucleic acid sequence, preferably a lacZ gene. Preferably, the lacZ gene includes the lacZ operator sequence. In a particularly preferred embodiment of the transgenic fish and method of the invention, the entire lacZ gene serves as the mutation target, and the lacZ operator sequence provides an opportunity for rapid and efficient purification of DNA. To detect mutations in the target gene, the plasmid is first rescued from the fish genomic DNA by extracting DNA from the cells of the transgenic fish or the transgenic fish cell, then the isolated DNA is fragmented, preferably by treatment with a restriction enzyme selected to excise the plasmid DNA from the chromosomal DNA. The plasmid-derived DNA is optionally isolated from the remaining genomic DNA, pref-

erably by contacting the restriction digest with an affinity support containing a binding material that preferentially binds to a nucleic acid sequence in the plasmid-derived DNA. In embodiments wherein the mutation target nucleic acid sequence includes the lacZ gene, the affinity support preferably includes a binding material that binds the lacZ operator, for example as β-galactosidase/LacI repressor fusion protein (also known as a LacZ/LacI fusion protein. DNA that does not bind to the binding material is removed, and the plasmid-derived DNA is eluted from the support. The plasmid-derived DNA is optionally circularized, then transformed into a bacterial host wherein a bioassay can be performed to detect the existence of a mutation in the mutation target nucleic acid sequence. In a preferred embodiment wherein the mutation target nucleic acid sequence comprises a lacZ gene, the bacterial host is preferably a restriction-negative, lacZ^{-}, galE^{-} bacterial host. In that system mutants are identified by culturing the transformed bacterium on a lactose-containing or lactose analogue-containing medium. Only bacteria which exhibit partial or no β-galactosidase activity can grow on this medium; growth is thus indicative of a mutation in the lacZ gene. If desired, the mutated target nucleic acid sequence can be sequenced to determine the type, location and extent of mutation, using, for example, a modified polymerase chain reaction (PCR) method or subdloning.

Various agents can be tested for mutagenic properties by exposing one or more transgenic fish of the invention to a suspected mutagen, then detecting mutations in the mutation target, e.g., the lacZ gene as described herein. In an analogous manner, the mutagenicity of various agents can be tested in vitro using cultured transgenic fish cells of the invention. The term "mutagen" is to be broadly understood as meaning any mutagenic or potentially mutagenic agent, event, or condition including a mutagenic chemical compound, such as a toxicant; radioactivity, including but not limited to alpha, beta, or gamma emissions from an radioisotope; electromagnetic radiation of any wavelength or frequency, such as x-ray, ultraviolet, or infrared radiation; exposure to a magnetic field or an electromagnetic field (EMF), and the like.

The transgenic fish or fish cell of the invention comprise one or more copies of the plasmid containing the mutation target nucleic acid sequence. A transgenic fish whose somatic and germ cells comprise at least one genomically integrated copy of the plasmid represents a preferred embodiment; more preferably, multiple copies of the plasmid are genomically integrated into the somatic and germ cells of the fish, most preferably in a head-to-tail orientation. The transgenic fish of the invention is preferably a teleost (bonecy) fish, but also includes a cartilaginous fish. Conveniently, the transgenic fish can be selected from among the well-known group of laboratory model fish which include medaka, zebrafish, mummichog, killifish, channel catfish, common carp and trout. Medaka, zebrafish or Fundulus spp. (for example, mummichog or killifish) are particularly preferred. In a highly preferred embodiment, the transgenic fish of the invention are the Japanese medaka (*O. latipes*) and the estuarine killifish (*F. heteroclitus*).

The invention also provides a genomically identical population of transgenic fish, each fish comprising one or more copies of the plasmid containing a mutation target nucleic acid sequence. A "genomically identical" population of transgenic fish is one wherein each fish has the same genomic DNA as the others in the population; this can also be referred to as a clonal population. Members of a genomically identical population are produced by cloning or inbreeding within the population rather than by mating with non-genomically identical fish (see, e.g., D. C. Streisinger et al., *Nature*, 291:293–296 (1981); W. Driever et al., *Trends Genet.*, 10 (5): 152–159 (1994)). Zygosity of the genomically identical population can be haploid, diploid or triploid. A genomically identical population is a single sex (unisex) population and can be male or female. A genomically identical female population can be produced, for example, by gynogenesis, wherein sperm is used to activate the egg but does not contribute genomic DNA to the developing organism (J H. Postlethwait et al., *Trends Genet.*, 13(5) :183–190 (1997)). Methods for making a genomically identical male population include heat shock, pressure shock, or irradiation, in combination with appropriate breeding techniques.

Although introduction of the plasmid into a host fish cell is not limited to any particular mode of delivery, microinjection is preferred. Microinjection is labor-intensive and time-consuming, but when practiced in accordance with the present invention results in a greater likelihood of introducing the DNA into the cell nucleus as opposed to the cytoplasm. While advances have been made relating to other methods of gene transfer such as electroporation of eggs (e.g., K. Inoue et al., *Cell Differ. Dev.* 29(2):123–128 (1990); F. Muller et al., *FEBS Lett.*, 324 (1):27–32 (1993); Y. Murakami et al., *J. Biotechnol.*, 34 (1):35–42 (1994); F. Muller et al., *Mol. Mar. Biol. Biotechnol.* 1 (4–5):276–81 (1992)) and sperm (Symonds et al., 1994; D. A. Powers et al., *Mol. Mar. Biol. Biotechnol.*, 1 (4–5):301–8 (1992)); particle gun bombardment (A. V. Zelenin et al., *FEBS Lett.*, 287 (1–2):118–20 (1991); liposomes (J. Szelei et al., *Biochem. J.*, 259 (2):549–53 (1989); and retroviral vectors (J. K. Lu et al., *Mol. Mar. Biol. Biotechnol.*, 6 (4):289–95 (1997)); these procedures only rarely result in transgen integration and germline transmission in fish (K. Inoue et al., *Cell Differ. Dev.* 29(2):123–128 (1990). Nonetheless, the invention is not intended to be limited to any particular method of introducing the heterologous DNA into the host organism. Preferably, the plasmid sequences are stably integrated into the host genome and are inheritable.

The plasmid used to transform the fish contains an assayable mutation target nucleic acid sequence. An "assayable" mutation target nucleic acid sequence is one wherein a mutated form of the nucleic acid sequence can be distinguished from the nonmutated form of the nucleic acid sequence, either directly or indirectly, as by using a laboratory assay or other detection procedure. For example, mutation of the mutation target nucleic acid sequence can result in a detectable change in the function of the nucleic acid sequence, or of a gene product encoded thereby. Preferably, the presence or absence of a mutation in the nucleic acid sequence is detectable by way of a chemical or biological assay. Preferably, the mutation target nucleic acid sequence includes a lad gene, a lacZ gene, a lac promoter sequence or an rpsL gene from *E. coli*. More preferably, it includes a lacZ gene, most preferably a lacZ gene that includes a lacZ operator. Inclusion of the lacZ operator allows rapid and efficient purification of the plasmid, as described above. Optionally the plasmid further includes an origin of replication (an ori), preferably a pBR322 ori, a selection marker gene, or both. A selection marker gene typically encodes a polypeptide which can confer a phenotypic characteristic to the transformed cell, such as antibiotic resistence, test compound metabolism, and the like. An ampicillin-resistance gene is an example of a suitable a selection marker gene. A representative preferred embodiment of the plasmid used to transform the fish according to the invention is pUR288

(FIG. 1). The plasmid pUR288 contains certain features common to preferred plasmids of the invention, namely the entire lacZ gene sequence (as the mutation target gene) including the lacO sequence (to allow affinity capture with the LacI repressor protein), together with the Co1EI and a marker gene, in this case the amp^R gene, to allow propagation and selection in *E. coli* (U. Rüther et al., EMBO J. 2(10):1791–1794 (1983)). The plasmid contains restriction sites, such as HindIII restriction sites in the case of pUR288, positioned to allow excision of the genomically integrated plasmid from the fish DNA. These restriction sites are located at or near each of the two ends of the linearized plasmid and thus flark the lacZ gene. The plasmid is incorporated directly into the fish DNA in a linearized form. Preferably, the plasmid vector used for transformation of the fish host cell comprises multiple end-to-end copies of the plasmid in a concatemeric configuration. The restriction sites are positioned between each copy of the plasmid, such that digestion with the appropriate restriction endonuclease releases monomeric plasmid sequences (single copies of the plasmid). It is not necessary, nor is it desirable, to insert the plasmid into a bacteriophage vector prior to insertion into the host's chromosomal DNA.

In a preferred embodiment, the plasmid is one that is suitable for transformation of bacteria, preferably *E. coli*. A host restriction-negative bacterial cell is preferred for use in the bioassay to detect the presence of mutations in the mutation target nucleic acid sequence. In a host-restriction negative bacterial cell, degradation of methylated plasmid DNA is prevented (the plasmid DNA is likely to have been methylated while in the fish host). When the mutation target nucleic acid sequence includes the lacZ gene, the preferred host cell is a host restriction-negative, $lacZ^{-}$, $galE^{-}$ *E. coli* strain. Non-mutant colonies do not survive in this preferred selection system; only those cells which have incorporated a mutated plasmid will grow after plating on the lactose-containing or lactose analogue-containing medium. *E. coli* cells that have incorporated a non-mutated plasmid will convert lactose or its analogue to galactose as a result of the presence of β-galactosidase. Further conversion of galactose is however not possible in the bacterial host due to the mutation in the galE gene. This leads to an accumulation of the toxic by-product UDP-galactose, whereby the host cell dies. Mutant colonies that produce no β-galactosidase survive because they do not convert the lactose or the lactose analogue to galactose. Mutants that exhibit residual levels of β-galactosidase activity also may grow to some degree on media containing lactose or a lactose analogue (M. Boerrigter, *Env. Mol. Mutagenesis* 32, 148–154 (1998) and are thereby distinguishable from nonmutants, which do not grow at all. It should be understood that the invention is not limited to the use of *E. coli* as the bacterial host; other types of bactena can also be used as long as suitable vector plasmids and host-restriction negative strains are available.

The plasmid is, of course, heterologous with respect to the host fish, the terms "heterologous" and "exogenous" being used herein interchangeably to denote nucleic acid sequences that have been inserted in to a host organism, but are not found in the normal genetic complement (i.e., genome) of the host organism. A gene that is heterologous with respect to an organism into which it has been inserted or transferred is sometimes referred to herein as a "transgene." A "transgenic" animal or host is an animal having one or more cells that contain exogenous (heterologous) nucleic acid sequences, including expression vectors.

Mutations in the mutation target nucleic acid harbored by the transgenic fish or fish cell of the invention are detected by first extracting DNA from the cells of the fish or fish cells, then the fragmenting the isolated DNA by treatment with a restriction endonuclease selected to excise the plasmid DNA from the chromosomal DNA. Techniques for genomic DNA extraction are well-known and any convenient method can be employed; however methods particularly suited for fish are performed using gentle tissue homogenation and proteolytic digestion at about 37° C. as described further in Example I. The restriction endonuclease recognizes restriction sites flanking the lacZ operon which have been engineered into the plasmid, so as to cut out or excise the DNA of the plasmid. It should be understood that the plasmid is considered to be excised if the restriction enzyme liberates the entire mutation target region (e.g., the lacZ gene); in other words, the restriction sites are at or near the two ends of the linearized plasmid and the plasmid thus released may be somewhat truncated in comparison to the plasmid originally transformed into the fish or fish cell. This could occur, for example, where the restriction endonuclease used to liberate the plasmid is different from the restriction endonuclease used to linearize the plasmid prior to introduction into the fish or fish cell. Preferably, the restriction endonuclease does not recognize a restriction site internal to the mutation target nucleic acid sequence. When pUR288 is used as the plasmid, a convenient restriction enzyme is Hind III.

If desired, the fragmented DNA present in the resulting restriction digest can be subjected to a separation protocol that separates the fragments containing the plasmid-derived mutation target nucleic acid sequence from the remaining fragments containing chromosomal DNA. Isolation of plasmid-derived DNA fragments can be effected using any convenient means such as affinity chromatography, gel electrophoresis, size exclusion chromatography, centrifugation or spin filtration, dialysis, and the like. Because of the efficiency of genomic integration, separation of the DNA fragments is optional, and the bacterial host can be transformed with either isolated or nonisolated plasmid-derived DNA fragments. When the mutation target nucleic acid sequence comprises the lacZ gene including the lacZ operator, the fragmented DNA is preferably and conveniently subjected to affinity purification using a lacZ-operator binding material. The lacZ operator binding material binds the lacZ operator present in the plasmid DNA. Affinity binding of the plasmid is preferably carried out on a solid support, more preferably on solid particles, most preferably on magnetic beads. The support is coated with a lacZ operator binding material. The lacZ operator binding material preferably comprises a protein. A lacZ operator binding protein can be an antibody with affinity for the lacZ operator, LadI repressor protein, or a LacI repressor fusion protein such as lacI repressor/β-galactosidase fusion protein or a LacI repressor/Protein A fusion protein. This material can be bound directly to the solid support but is preferably bound indirectly to the solid particles. Beads and reagents are commercially available from Dynel (Oslo, Norway). Anti-βgalactosidase and LacI repressor/β-galactosidase fusion protein are typically successively bound to the solid particles. After contact of the organism's genomic DNA with the solid support and removal of the unbound DNA fragments, the bound DNA fragment is eluted from the support using one or more agents having greater affinity for the LacI repressor or the lacZ operator than the LacI repressor and the lacZ operator have for one another. Preferably, plasmid DNA is released from the solid support using isopropyl β-D-thiogalactoside (IPTG), which eliminates the binding between the LacI repressor and the lacZ

operator. Affinity purification typically removes essentially all the excess chromosomal DNA, and circularization of the plasmid can be efficiently performed thereafter in a small volume.

Prior to transformation into the bacterial host, the plasmid-derived DNA is preferably circularized by ligation, for example enzymatic ligation using T4 ligase. The plasmid-derived DNA is then transformed into the bacterial host using methods well-known in the art. Typically, the circularized plasmid is electroporated into the bacterial host. Mutants are identified by selective growth on the culture medium, as described herein.

Mutation Analysis

The transgenic fish of the invention can be used for mutagenesis testing by exposing it to potential mutagenic agent. A mutated target sequence can be subjected to nucleic acid sequencing to determine the mutation spectrum characteristic of a particular mutagen, or of a particular tissue, or of the action of a particular mutagen on a particular tissue. Mutation spectra reflect the frequency of certain specific types of mutations in a population of mutants. Types of mutations include, for example, nucleotide transitions (G/C to A/T and A/T to G/C), nucleotide transversions (G/C to T/A, G/C to C/G, A/T to T/A, and A/T to C/G), and frameshift mutations (e.g., +1, +2, −1 and −2). Alternatively, mutations can be identified using single nucleotide polymorphism analysis, or any other method known in the art for identifying or detecting single site mutations, insertions, deletions and frameshifts.

The mutation spectrum of a population of mutants can provide much useful information. The spectrum may be characteristic of a particular mutagen or class of mutagens, and can help identify the nature of the mutagenic compound. In some instances, a change in the mutation spectrum, relative to the mutation spectrum of a control group, may be evident even though the differences in mutation frequencies (experimental vs. control) are not statistically significant. Mutation spectra analysis may also provide insight into the possible mechanism(s) of action of a particular mutagen, in different organs and tissues. In addition, mutation spectra analysis can yield information about the sensitivity of different organs or tissues to a particular mutagen.

Advantages of a Plasmid-based Mutation Detection System

A plasmid-based mutation detection system has a distinct advantage over other transgenic mutation detection systems in that it is able to detect a wide spectrum of mutations, particularly large sequence deletions or rearrangements induced by clastogenic agents such as radiation. Plasmid recovery is not as size-dependent as it appears to be the bacteriophage-λ sequences, due to λ's special requirements for in vitro packaging. Thus, large sequence deletions extending into the flanking regions of the host chromosomal DNA are detectable in the plasmid-based system. As a result, all types of DNA rearrangements, including the large-scale structural DNA alterations induced by such agents as ionizing radiation, can be detected with the lacZ mutation target in this system, not just point mutations (J. Gossen et al., Mut. Res. 331:89–97 (1995)). There are currently no other transgenic in vivo mutation detection systems that have demonstrated comparable capabilities.

The small size of a plasmid vector compared to a bacteriophage vector also provides several important advantages over bacteriophage-based mutation detection systems. Its smaller size facilitates genomic integration of multiple copies of the plasmid. For example, pUR288 is about 5.5 kb, approximately 1/10 the size of the bacteriophage λ (~50 kb). The transgenic fish of the invention thus preferably carries multiple copies of the plasmid sequence which can be transmitted to its offspring. The small size of the plasmid also greatly simplifies requirements of DNA extraction, making recovery of the mutation marker more efficient. Incorporation of the lacO sequence in the plasmid vector in accordance with the present invention allows plasmid DNA to be purified from restriction-enzyme digested genomic DNA of the fish in a single step using high capacity LacI repressor magnetic beads, further increasing yields of the mutation marker. For example, pUR288 recovery is over 25 times more efficient than λ sequence recovery (J. Gossen et al., Mut. Res. 331:89–97 (1995)). In addition to detecting a wider range of mutations, the plasmid-based system of the present invention is thus also more cost-effective than bacteriophage-based systems, and mutations can be accurately determined based on large numbers of colony forming units using small amounts of tissue. In addition, mutations are detected by a selective rather than a color procedure which reduces ambiguity in analyses.

Host Organism

There are many advantages to the use of fish to detect mutagenic agents or events. Fish are environmentally relevant models for health risk assessment of aquatic and marine systems. There is also increasing appreciation of their suitability for biomedical applications. The fish is an alternative, nonmammalian animal model that can be used to refine, reduce or replace traditional animal models used in research and testing. Fish are easily handled, manipulated, and observed without compromising natural development, and present opportunities for studies on multiple exposure routes via aqueous media. They exhibit excellent agent-specific responses to a variety of toxicants. Teleost fish, also known as the modern boney fishes, constitute the largest and most diverse division of vertebrates, with over 20,000 known species. Their diversity and phylogenetic positions make them ideal for comparative toxicological studies, which may allow more insight into basic mechanisms than would studies limited to mammalian models alone (D. A. Powers, *Science* 246:352–358 (1989)). Certain teleost fish, commonly referred to as laboratory aquarium fish or laboratory model species, have been extensively studied in research settings and are thus especially well-suited as transgenic hosts. Laboratory model species include, but are not limited to, medaka (*Oryzias latipes*), zebrafish (*Danio rerio*), mummichog (*Fundulus heteroclitus*), killifish (Fundulus spp.), channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*) and trout (*Salmo gardneri*). Cartilagenous fish such as sharks and rays, also known as chondrichthyes fish, are also suitable transgenic host organisms.

In addition to playing an important role in comparative mutagenesis studies, it is anticipated that the transgenic fish models may ultimately facilitate a broad range of exposure regimens such as long-term low-dose chronic exposures, controlled field-based in situ exposures or large-scale mesocosms, that were previously difficult or impossible to perform. Fish can be conveniently used for zygote-to-adult exposure studies, and offer flexibility in study designs related to numbers of exposure groups and exposure schedules.

Fish eggs are relatively large, abundant, and often translucent, and can be fertilized in vitro. The resulting embryos are easily maintained and develop externally, obviating the need for reintroduction of the embryo into a receptive female. In addition, laboratory analyses can generally be performed more rapidly and at lower cost compared to rodent assays.

Heterologous genes have been introduced into fish beginning in 1984. Among these heterologous genes that have been introduced into fish include genes that code for growth hormones (human, rat, rainbow trout), chicken delta-crystalline protein, *E. coli* β-galactosidase, *E. coli* neomycin-resistance, and anti-freeze protein. However, numerous problems have been encountered in producing transgenic fish having stable, inheritable genomically integrated transgenes. For example mosaicism, which results from the failure of the transgene to integrate into the host's genomic DNA at the first mitotic division, is a common problem in the creation of transgenic fish. Mosaic organisms do not contain the transgene in every somatic and germ cell, and thus may not be capable of producing transgenic offspring. Mosaicism is presumed to arise from fact that microinjection of heterologous DNA into a fish embryo often delivers the heterologous DNA to the cytoplasm rather than the cell nucleus.

Despite the evolutionary distance between fish and humans, there is increasing evidence for correlation between environmentally-induced fish and human diseases. Fish can be exposed to different concentrations of known or suspected toxicants and can provide fundamental information related to substance toxicity and carcinogenicity/mutagenicity. The use of fish in carcinogenesis research, in particular, has received considerable attention related to the potential of fish for identifying and predicting human health effects (W. F. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor and Francis. 421–446 (1995); J. D. Hendricks, In L. J. Weber, ed., Aquatic Toxicology, Raven Press, New York. 149–211 (1982); J. J. Black, In J. Saxena, ed., Hazard Assessment of Chemicals: Current Developments, Vol. 3. Academic Press, New York. 181–232 (1984); C. D. Metcalfe, *CRC Rev. Aquat. Sci.* 1:111–129 (1989)). The fact that many fish species appear to be sensitive to the carcinogenic effects of certain chemicals while having low spontaneous rates of neoplasia supports the use of fish in various assays as alternatives or supplements to rodent chronic bioassays (G. D. Bailey et al., *Environ. Health Perspect.* 71:147–153 (1987); T. Ishikawa et al., *J. Toxicol. Environ. Health* 5:537–550 (1979); P. Masahito et al., *Jpn. J. Cancer Res.* 79:545–555 (1988)).

The genetics, developmental biology and embryology of medaka (*Oryzias latipes*) are well-documented, and specific developmental stages have been extensively characterized (T.O. Yamamoto, Medaka (killifish): Biology and Strains. Keigaku Publishing Co., Tokyo, Japan. (1975)). Medaka is typically used to study aspects of various diseases in which large numbers of experimental organisms are required, such as in low-dose risk assessment, as well as to examine factors that only slightly increase hazard exposure risk (W. W. Walker, W. E. Hawkins, R. M. Overstreet, and M. A. Friedman, "A small fish model for assessing cancer risk at low carcinogen concentrations," *Toxicologist* 302 (1992)). The use of medaka in biomedical research, especially as a carcinogenesis model related to the potential for identifying and predicting human effects from toxicant exposure, has received considerable attention in recent years (W. E. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor and Francis. 421–446 (1995); J. D. Hendricks, In L. J. Weber, ed., Aquatic Toxicology, Raven Press, New York. 149–211 (1982); J. J. Black, In J. Saxena, ed., Hazard Assessment of Chemicals: Current Developments, Vol. 3. Academic Press, New York. 181–232 (1984); and C D. Metcalfe, *CRC Rev. Aquat. Sci.* 1:111–129 (1989)). The sensitivity of medaka to many carcinogens, the availability of specimens, and the degree of control that can be maintained over extraneous factors all contribute to this small fish being one of the most widely used species for studies in comparative toxicology (W. E. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor and Francis. 421–446 (1995)), the biology of hepatic neoplasia (D. E. Hinton et al., *Aquat. Toxicol.* 11:77–112 (1988)), oncogene activation (R. J. Van Beneden et al., *Cancer Res.* 50:5671s–5674s (1990)), DNA repair (T. Ishikawa et al., *Natl. Cancer Inst. Monograph* 65:35–43 (1984)), and mutagenesis (R. N. Winn et al., *Marine Environ. Res.* 40(3):247–265 (1995)).

Medaka offer numerous advantages for transgenic development such as small size (about 2.5 cm), relatively short generation time (1–2 months), and prolific capacity to reproduce (more than 3,000 eggs/female in a single breeding season). Spawning can be induced year-round by maintaining breeding stocks at 25–28° C. and eggs usually hatch in 10 days at 25° C. Eggs are translucent, which greatly facilitates the positioning of fine glass needles for DNA microinjection. Medaka was the first transgenic fish species produced to demonstrate successful foreign gene expression (K. Ozato et al., *Cell Differ.* 19:237–244 (1986)). Subsequently, numerous transgenic medaka have been produced that carry a variety of transgenes (e.g. K. Inoue et al., *Cell Differ. Dev.* 27(1):57–68 (1989); E. Tamiya et al., *Nucleic Acids Res.* 18:1072 (1990); K. Inoue et al., *Cell Differ. Dev.* 29(2):123–128 (1990); J. Lu et al., *Mol. Marine Biol. and Biotechnol.* 1(4/5):366–375 (1992); H. J. Tsai et al., *Mol. Mar. Biol. Biotechnol.* 4(1):1–9 (1995); R. N. Winn et al., *Marine Env. Res.* 40(3):247–265 (1995)). However, transgenic Fundulus have been produced only once (R N. Winn et al., *Marine Environ. Res.* 40(3):247–265 (1995)).

As noted above, the invention is intended to further encompass progeny of a transgenic fish containing a genomically integrated plasmid comprising a mutation target nucleic acid sequence, as well as transgenic fish derived from a transgenic fish egg, sperm cell, embryo, or other cell containing a genomically integrated plasmid comprising a mutation target nucleic acid sequence. "Progeny," as the term is used herein, can result from breeding two transgenic fish of the invention, or from breeding a first transgenic fish of the invention to a second fish that is not a transgenic fish of the invention. In the latter case, the second fish can, for example, be a wild-type fish, a specialized strain of fish, a mutant fish, or another transgenic fish. The hybrid progeny of these matings have the benefits of the transgene for mutation detection combined with the benefits derived from these other lineages.

EXAMPLES

The following examples, while exemplary of the present invention, are not to be construed as specifically limiting the invention. Accordingly, variations and equivalents, now known or later developed, that would be within the purview of one skilled in the art are to considered to fall within the scope of this invention.

Example I

Creation of a Transgenic Fish

Preparation of plasmid DNA

The pUR288 plasmid DNA was provided by Michaël E.T.I. Boerrigter (Leven, Inc., Bogart Georgia). In order to enhance integration of multiple copies of the plasmid

pUR288, the DNA was cut with a restriction enzyme (Hind III; New England BioLabs), and then ligated into a ladder of multiple copies (4 minute ligation at 4° C. with T4 ligase (New England BioLabs), 0.5 mm ATP, 0.06 units/μl ligase, 1×ligase buffer). DNA was brought to 750 μl volume with TE (TE: 10 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA)) and extracted twice with phenol/chloroform, once with chloroform, and precipitated with 100% ethanol. Following centrifugation, the pellet was washed with 70% ethanol, dried, and dissolved in TE at 100–200 ng/μl. Prior to microinjection, the DNA was diluted to 50–100 ng/μl in 5T.1E solution (5 mM Tris; 0.1 mM EDTA) and dialyzed on a filter over 5T.1E for 40 minutes (0.025 micron pore size, Millipore Corporation).

Microinjection of Fish Embryos

Fish embryos were microinjected generally in accordance with Winn et al. (*Marine Environ. Res.*, 40(3):247–265 (1995)), but with the modifications described herein. For medaka, in order to maximize incorporation of the gene and reduce the degree of mosaicism in the founders, fertilized eggs at the one-cell stage were collected by removing egg masses from the vent of the female fish beginning 2 hours prior to the onset of a 16 hour light-cycle and every 10–15 minutes thereafter. The gene transfer method was optimized by rigidly controlling the timing of the injection at the earliest 1-cell stage of development of the fish, in most cases within 5 minutes of fertilization. The embryos were individually separated by removing the entangling chorionic fibrils and examined to verify the one-cell stage of development for efficient gene transfer. The one-cell embryos were placed within a watch-glass filled with 1‰ (parts per thousand salinity) seawater to better visualize the penetration of the injection needle and to reduce the incidence of fungal infection. Fundulus eggs were stripped from the females immediately prior to injection, placed in a watch glass and fertilized with sperm collected by applying pressure to the abdomen of fertile males.

Injections were performed with the aid of a dissection microscope, micromanipulators, and an N_2 pressurized gas injection apparatus (PLI100 Medical Systems Corp., Greenvale, N.Y.). The embryos were held in place with a capillary pipette (25 μm) secured by a micromanipulator. Another capillary pipette pulled to a fine tip (1–2 μm), secured by a micromanipulator, and attached to a gas injection apparatus served as the injection needle. The DNA solution was injected through a continuously flowing pipette into the cytoplasm of the one-cell embryo, or through the micropyle when visible. This is in acontrast to the technique used in rodents, wherein the DNA solution can be directly injected into the rodent cell nucleus. It is believed that injection through the micropyle is preferable since introduction into the cytoplasm may increase the likelihood of degradation of the DNA construct and, more important, may give rise to mosaic (or chimeric) integration of the gene in the tissues of a resultant transgenic fish. That is, not all of the cells will have the heterologous DNA integrated chromosomally. Mosaic integration of the transgene in transgenic fishes is very common and is problematic because germ-line transmission is not guaranteed even if DNA extracted from a fin clip (the typical assay for integration) indicates the founder fish carries the gene. The flow rate and the total amount of solution injected was controlled by adjusting the pressure of the gas and the duration of the injection to permit injection of approximately 5–20 nl DNA solution. Medaka embryos were transferred to 20 mm petri dishes and incubated at 26° C. in sterile culture water until hatching in approximately 10 days. Fundulus embryos were placed on blotting pads on petri dishes moistened with seawater for about 14 days until hatching.

Analyses of Presumptive Transgenic Fish

Fifteen founders were obtained from the 139 medaka tested (11%), and seven out of the 15 exhibited germline transmission (47%). In the Fundulus, out of 116 fish tested, 25 were positive (22%). Thirteen of the 25 fish were tested for germline transmission, and 3 of the 13 showed positive germline transmission (23%). Of the three Fundulus showing germiline transmission, one exhibited 9% transmission to its offspring, another exhibited had 11% transmission, and the third exhibited 41% transmission.

Fish tissues appear to pose a significant problem related to efficient recovery of bacteriophage and plasmid-based vectors for mutation detection. The extraction of high quality and high molecular weight genomic DNA is very important to the efficient recovery of the shuttle vector from transgenic rodent tissues. However, repeated attempts to recover shuttle vectors from transgenic fish tissues using the procedures developed for rodents have in the past been unsuccessful. Accordingly, genomic DNAs were isolated from fish tissues as generally described in R. N. Wirn et al. (*Marine Environ. Res.* 40(3):247–265 (1995)) with modifications as described herein.

Transgenic fish that demonstrated stable germ-line transmission were selected for the analysis of recovery and spontaneous mutation frequency of the plasmid shuttle vector. In addition, standard procedures for isolating genomic DNA from rodents were altered so as to insure isolation of high quality genomic DNA sufficient for recovering shuttle vectors from the fish, as described below. Specifically, transgenic F_1 generation fish (4–6 weeks old) were disaggregated with a dounce homogenizer containing douncing buffer (1×SSC, 1% SDS), digested with proteinase K (GibcoBRL) (37° C.) for about 1½ hours, twice extracted with equal volumes of phenol/chloroform, followed by chloroform, and precipitated with 2 volumes 100% ethanol. Precipitated DNA was spooled with a flame-sealed Pasteur Pipette, dried in air, and resuspended in 50–100 μL TE buffer (Tris 10 mM, EDTA, 1 mM, pH 7.5). Importantly, this protocol incorporates a shorter digestion time (typically about 1½ hours; sometimes up to about 2½ hours) at a lower temperature (37° C.) compared to the standard procedure used to isolate genomic DNA from mice (wherein the digestion is carried out at 50° C. for 3 hours), thus enhancing the recovery of assayable genomic DNA from the fish.

It should be noted that this DNA extraction method can be used to recover DNA directly from an organ or a tissue of a fish. The protocol is typically carried out using reduced volumes (since the amount of biological material to be extracted is reduced), and the ethanol-precipitated DNA can be isolated by centrifugation rather than by spooling. This method offers great benefit in that it allows for the study of tissue-specific mutation frequencies.

Screening for genomic integration of the pUR288 in the fish was performed by using polymerase chain reaction (PCR) methods. Amplified products (approximately 200–800 kb) were generated using several lacZ primers. The standard amplification temperature profile were as follows: denaturing 95° C., 30 seconds; annealing 60° C., 30 seconds; extending 72° C., 60 seconds. Electrophoresis of the products on an agarose gel was used to confirm amplification of the DNA fragment of appropriate size.

The zygosity of transgenic F2 generation fish was determined using quantitative PCR methods followed by verification using standard PCR analysis of DNA obtained from the offspring produced from breeding with wild-type fish in

which the transmission of the plasmid to ~100% of the offspring confirmed homozygous parentage while a transmission frequency of ~50% demonstrated a hemizygous parent.

The methods for the quantitative PCR using the TaqMan system (Applied Biosystems, Calif.) entailed preparing the genomic DNA from transgenic fish in replicate at three concentrations (10 ng, 100 ng, 1000 ng). No amplification and no template DNA samples were prepared either with DNA from non-transgenic fish, or with sterile water in the place of DNA in the reagent mixture. Serial dilutions (10 ng, 100 ng, 1 ug) of genomic DNA from hemizygous and homozygous transgenic fish were used-to generate a standard curve. The forward and reverse primers consisted of a 19 mer oligonucleotide (5'-CCGCTGATCCTTTGCGAAT-3') (SEQ ID NO:1) and a 18 mer oligonucleotide (5'-CGAAGCCGCCCTGTAAAC-3') (SEQ ID NO:2), respectively, that annealed within the lacZ gene sequence to generate a PCR product. The probe that annealed within the PCR product consisted of a 31 mer oligonucleotide (5'-TGCCAGTATTTAGCGAAACCGCCAAGACTGT-3') (SEQ ID NO:3) with FAM (6-carboxy-fluorescein) as the reporter dye linked to the at 5' end, and TAMRA (6-carboxy-tetramethylrhodamine) as the quencher dye attached at the 3' end. The amplification conditions were as follows: 100 ng transgenic fish genomic DNA; 4 mM $MgSO_4$; 200 M dATP, dCTP, dGTP; 400 uM dUTP; 125 M probe; 0.625 U Amplitaq Gold™ M polymerase; 0.25 U Amerase UNG; and 1× of supplied buffer (Perkin Elmer Cetus). Using a two stage amplification profile, stage I consisted of an initial incubation of 2 minutes at 50° C., 10 minutes at 95° C.; followed by stage II with 15 seconds at 95° C., 1 minute at 60° C. for 30 cycles. A hold cycle at 25° C. was used following completion of amplification.

Example II

Determination of Spontaneous Mutation Freguency in the Target Gene

Plasmid rescue and mutation frequency analysis involved the preparation of the magnetic beads, isolation of the plasmid DNA from fish genomic DNA, electroporation of the plasmid into *E. coli*, and mutation frequency quantitation on selective plates.

The methods for preparation of the LacI repressor protein magnetic beads essentially followed previously described protocols (Gossen et al., *BioTechniques* 14, 624–629 (1993)); J. Gossen et al., *Mut. Res.* 331:89–97 (1995); see also M. Boerrigter, *Env. Mol. Mutagenesis* 32, 148–154 (1998); M. Boerrigter et al., *Nature* 377:657–659 (1995); M. Dollé et al., *Mutagenesis* 11:111–118 (1996); Boerrigter et al., *Environ. Mol. Mutagen.* 29:221–229 (1997) for additional experimental details and variations of the procedure). One mL Dynal M450 magnetic beads (Dynal AS, Oslo, Norway) coated with sheep anti-mouse IgG (4×10^8 magnetic beads/mL), were added to 130 μl anti-β-galactosidase (2.3 mg/mL) and incubated 45 minutes at room temperature. The magnetic beads were pelleted on a magnetic stand (Dynal) and the supernatant removed. The beads were washed 3 times for 5 minutes in 1 mL phosphate-buffered saline (PBS)/0.1% bovine serum albumin (BSA). After the last wash, the pellet was resuspended in 900 μl PBS/0.1% BSA, and 75 μl β-galactosidase/LacI repressor fusion protein (2.0 mg/mL, Promega) was added and incubated for 2 hours at room temperature. The β-galactosidase/LacI repressor fusion protein is also commercially available from Dynal (Oslo, Norway) and Leven (Bogart, Ga.). Beads were pelleted and washed 2 times with 1 volume PBS buffer and once with storage buffer (20 mM Tris-HCl, pH 7.6, 2 mM EDTA, 5% glycerol, 1 mg/mL acetylated BSA, 0.02% Na-azide). Beads were resuspended in 1 mL storage buffer and stored at 4° C. until needed.

Transgenic fish genomic DNAs were digested with the restriction enzyme HindIII then diluted. The final recovery of about 10–50 μg fish DNA. Restriction digest of a non-mutated plasmid generates an approximately 5.5 kb fragment containing the complete pUR288 plasmid. The DNAs were added to LacI repressor magnetic beads and incubated for 1.5 hour at room temperature. Beads were washed twice with Tris-HCl, pH 7.6, 2 mM EDTA, 5% glycerol, resuspended in IPTG elution buffer (10 mM Tris-HCl, pH 7.6, 2 mM EDTA, 20 mM NaCl, 135 μg/mL IPTG) and incubated for 20 minute. The linear pUR288 DNA was circularized using T4 DNA ligase (GibcoBRL, in GibcoBRL T4 ligase buffer, which includes adenosine triphosphate [ATP] at a concentration of 0.1 mM) in a 2 hour incubation at 16° C., precipitated with 5 μg glycogen, ⅛o volume 3 M sodium acetate, pH 4.9 and 2.5 volumes cold 100% ethanol and resuspended in TE buffer.

In other experiments, the magnetic beads were prepared as described in M. Dollé et al. (*Mutagenesis* 11, 111–118 (1996); also V g et al., *Technologies for Detection of DNA Damage and Mutations*, Plenum Press, N.Y. (1996)), and the modified plasmid rescue protocol as described therein was used.

Briefly, the above protocol is streamlined by combining the HindIII digestion and the incubation with the fusion protein-coated magnetic beads simultaneously, using as a binding/excision buffer containing 10 mM Tris-HCl, 1 mM EDTA; 10 mM $MgCl_2$, 5% glycerol, pH 6.8, which also included the HindIII; a preferred binding excision buffer contains Mg^{2+} from about 6–12 mM, and is about pH 6.5–7.5. IPTG elution was followed by a second HindIII digestion and the ligation, which were all done in the presence of the beads in the same tube.

Electroporation of plasmids was performed by adding circularized plasmid (1–2 μl) to electrocompetent *E. coli* C lacZ$^-$, galE$^-$ host cells, then electroporating at 25 μF. and 2.5 kV, 200 Ω using 0.2 cm cuvettes. After electroporation, SOC medium (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added. The cells were incubated in a shaking water bath (225 rpm) for 1 hour at 37° C. This strain is host-restriction negative, which prevents degradation of methylated plasmid DNA. The mutation in gale facilitates the selection of lacZ mutants on a medium containing lactose or a lactose-analogue such as phenyl-β-D-galactoside (βgal). GalE mutants lyse in the presence of galactose because, lacking the enzyme UDPgal 4-epimerase, they accumulate toxic amounts of UDP-galactose. Thus nonmutant host cells, which produce high β-galactosidase levels and hence convert p-gal to galactose, cannot grow, whereas the mutant host cells produce little or no β-galactosidase and thus form colonies.

Rescue efficiencies and mutation frequencies were determined substantially as described in M. Boerrigter (*Env. Mol. Mutagenesis* 32, 148–154 (1998)). Single mutant CFUs were transferred to the wells of a 96 well round bottom polystyrene cell culture plate (CoStar, Cambridge, Mass.) containing 100 μL LB medium supplemented with 75 μg/mL ampicillin and 25 μg/mL kanamycin (Sigma Chemical Co., St. Louis, Mo.) and grown for 3 hours at 37° C. To determine the sensitivity for p-gal and β-galactosidase activity,

respectively, 2 μL of each cell culture was transferred to LB-agar plates, containing 75 μg/mL ampicillin, 25 μg/mL kanamycin, and either 0.3% p-gal (Sigma Chemical Co., St. Louis, Mo.) or 75 μg/mL 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-gal; Promega, Madison, Wis.).

Mutant frequencies were determined as the ratio between the number of colonies on selective (p-gal) plates versus the number of colonies on non-selective (X-gal) plates, times the dilution factor. A minimum of 200,000 cfu were analyzed in each assay. Variations of the assay protocols can be found in M. Dolle et al. (*Mutagenresis* 11, 111–118 (1996)), and M. Boerrigter et al. (*Nature* 377, 657–659 (1995)). Plasmid recovery and spontaneous mutation frequency for the seven gemiline founders are set forth in Table I:

TABLE I

Plasmid recovery and spontaneous mutation frequency in medaka

Germline Transmitting Founders	Transmission Rate	Recovery	Mutation Frequency
#9*	16%	5.82×10^5	112×10^{-5}
#25	3%	n/a	
#47	6%	n/a	
#54*	10%	n/a	n/a
#68*	38%	12×10^5	24×10^{-5}
#127*	44%	40×10^5	9.6×10^{-5}
#130	n/a	n/a	

*Producing F2s

Some transformed cells showed blue colors of varying intensity on the X-gal plates, suggesting that some of the mutants retained some β-galactosidase activity. Boerrigter has reported (Env. Mol. Mutagenesis 32, 148–154 (1998)) that about 21% of mutant CFUs displayed a discernable β-galactosidase activity that was less than that exhibited by nonmutant CFUs. It was found that residual β-galactosidase activity correlated with single base pair substitutions in the lacZ gene. Thus, this positive selection system has the capacity to detect a broad range of mutations, ranging from large size changes, including deletion and additions, that inactive lacZ completely to single base pair changes that only partially inactivate the lacZ gene.

Example III

Mutation Analysis

Mutants can be screened or analyzed using any suitable technique known in the art. Preliminary screening can be accomplished, for example, by assaying mutants for residual β-galactosidase activity (Example II; M. Boerrigter, *Env. Mol. Mutagenesis* 32, 148–154 (1998)), which is indicative of mutations that produce a partially gene product (e.g., single base pair mutations). Mutants can also be subjected to thermal gradient gel electrophoresis, a 2D gel electrophoresis technique that is useful in distinguishing plasmid size changes (deletions, insertions) from point mutations. By providing information about the nature of the mutation, preliminary screening techniques can make subsequent sequence analysis of a selected mutant more efficient by narrowing down the number of primers needed to successfully amplify the mutated region of the target lacZ gene.

Selected mutations can analyzed by sequence analyses of the lacZ target gene using linear amplification sequencing in which the components of a chain-termination sequencing reaction are cycled through a standard PCR temperature profile. The mutation target (lacZ) is large, thus a suite of primers is typically used to generate amplification products of a convenient size for sequencing. The lacZ template DNA are prepared for sequencing by PCR using the mutant plasmid directly as the template or by further purification. The sequencing reaction mixture (template DNA, lacZ primers, sequencing buffer, radioactive label, polymerase, water and DMSO) is cycled through a temperature profile of denaturation 95° C. 5 minutes, annealing 60° C., 30 seconds; and extension 72° C., 60 seconds. The products (2–4 μl) are loaded on a standard sequencing gel and analyzed for sequence differences. Comparisons are made of the type, number and percentages of mutations.

Mutation analysis is useful to determine the mutation spectrum characteristic of a particular mutagen, or a particular tissue, or of the action of a particular mutagen on a particular tissue.

Example IV

Mutagen Exposure Experiments

Radiation

Transgenic fish are exposed to a mutagen using a generalized exposure regimen adapted from a protocol used previously with transgenic mice carrying the plasmid vector pUR288 as the mutation target (J. Gossen et al., Mut. Res. 331, 89–97 (1995)). Radiation exposure experiments can be performed on the fish at the University of Georgia's Center for Applied Isotope Studies using an in-house ^{60}Co irradiation source (Atomic Energy of Canada, Ltd., Gammacell 200; having a total activity of 1.22×10^{13} Bq). To assess the effects of exposure to X-rays, X-ray treatment will be performed by whole body irradiation using a University of Georgia X-ray source.

Chemical Mutagens

N-ethyl-N-nitrosourea (ENU) is a well-characterized mutagen and carcinogen that acts by direct ethylation of oxygen and nitrogen in the bases of DNA (B. Singer, *Nature* 264:333–339 (1976)); B. Singer et al., *Nature* 276:85–88 (1978)). ENU is a useful agent for the study of the relationship of mutation to DNA repair, replication, adduct persistence, and cell differentiation (J G. Burkhart et al., *Mutation Res.* 292:69–81 (1993)). A limited study of ENU-induced mutation has been previously performed using medaka (A. Shimada et al., *Zoological Sci.* (Tokyo) 8(6): 1127 (1991); A. Shimada et al., *Zoological Sci. (Tokyo)* 7(6):1053 (1990)).

Dimethylnitrosamine (DMN) is a methylating agent and potent liver carcinogen in mice (International Agency for Research on Cancer (IARC), IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Vol. 7, IARC, Lyon. 253p (1974)). Since cell proliferation is an important parameter for the induction of mutations, it is important to consider the influence of fixation time of adducts on the induction of mutations. DMN has been used as a representative mutagen which forms methylated DNA adducts in transgenic mice (J. C. Mirsalis et al., *Mutagenesis* 8:265–271 (1993)). DMN is among the nitrosamines that have induced hepatocarcinogenesis in fish with progressive stages similes to those characterized in rodent hepatic neoplasia (W. E. Hawkins et al., Chapter 14 in G. M. Rand, ed., Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor and Francis. 421–446 (1995)).

Exemplary Exposure Regimen

Fish mutagen exposure is performed by using protocols employed in previous transgenic rodent studies (e.g., M. J. Dycaico et al., *Mutation Res.* 307:461–478 (1994); B. J.

Rogers et al., *Mutation Res.* 327:57–66 (1995)). Specifically, one regimen consists of a single-pulse 1 to 4 hour exposure; another consists of a multiple-pulse exposure regimen consisting of 2 pulses for a 4 hour exposure for 7 days. Prior to final exposure, range-finding assays for each of the two model compounds are conducted wherein fish are exposed in single-pulse and multiple-pulse treatments over a range of mutagen concentrations to determine the upper exposure concentration expected to produce minimal or zero lethality (about 50% of the lowest exposure concentration at which deaths occur). The range finding trials in the present example are 0, 25, 50 and 100 ppm of ENU or DMN. In previous mutagen exposure studies using λLIZ transgenic fish, doses of about 0.5 mM (60 ppm) of ENU (LD_{50} ~350 mg/kg) were used to obtain a two-fold mutation frequency induction, and doses of about 120 ppm of ENU were used to obtain four-fold mutation frequency induction in medaka.

Fish are placed in replicate 50 mL glass test chambers (10/chamber). The mutagen solutions, at concentrations determined by dilution factors, are added to water immediately prior to the initiation of exposure. Toxicant-free treatments accompany all exposures as controls. For the multiple-pulse regimen, fish are transferred and held in clean water to await the next exposure. The fish are not fed during the exposure period. During the exposure phase, fish are monitored regularly for any visual signs of distress. Any dead or moribund fish are removed.

Following the final exposure series, fish are rinsed and transferred to grow-out aquaria for a prescribed expression time. During this time fish are held in aquaria in toxicant-free water at 26° C. on a 12:12 hour light:dark regime and fed twice daily. The fish are visually monitored at least twice daily during feeding. Any fish that have died, or that exhibit abnormal swimming behaviors or other visible signs of distress, are removed from the aquaria. Fish that show apparent formation of external neoplasms are removed, sacrificed, and saved for furthers analyses, if desired.

The influence of expression time on the mutation frequency is evaluated by sampling fish at 5, 10, 15 and 30 days following exposure. Expression time, or fixation time, is defined as the time allowed between dosing and sacrificing the animals for mutation assays. Some expression time is required, especially after single-dose administrations, to permit uptake and distribution of the chemical, metabolic activation to a DNA-reactive form, formation of adducts, and at least one cell division to "fix" the adduct as a heritable mutation. Although there is no data currently available on mutations rates, DNA repair, or cell proliferation in transgenic medaka, it is believed that a long expression time (>7 days) would allow adequate time to either repair DNA adducts or fix adducts as mutations. This reduces the possibility the DNA adducts will be mutated by the host *E. coli* by decreasing the number of DNA adducts present on the recovered target DNA.

Upon termination of the expression phase, fish are euthanized by overdose of MS-222 (tricaine methanesulfonate). Selected tissues are dissected and flash frozen in liquid nitrogen and stored at −70° C. Genomic DNA is isolated, the heterologous nucleic acid sequence is rescued, and mutation frequency is analyzed as in Example II.

The complete disclosures of all patents, patent applications, publications, database entries, submissions and deposits, including GENBANK deposits and the descriptive information associated therewith, and other documents cited herein are fully incorporated herein in their entireties by reference as if individually incorporated. Various modifications and alterations of this invention will become apparent to those skilled in the art without departing from the scope and spirit of this invention, and it should be understood that this invention is not to be unduly limited to the illustrative embodiments set forth herein.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 3

<210> SEQ ID NO 1
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
oligonucleotide primer

<400> SEQUENCE: 1

ccgctgatcc tttgcgaat 19

<210> SEQ ID NO 2
<211> LENGTH: 18
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence:
oligonucleotide primer

<400> SEQUENCE: 2

cgaagccgcc ctgtaaac 18

<210> SEQ ID NO 3

-continued

<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: oligonucleotide probe

<400> SEQUENCE: 3

tgccagtatt tagcgaaacc gccaagactg t 31

What is claimed is:

1. A method for detecting mutations in the DNA of a transenic fish comprising:

providing a transgenic fish, wherein the DNA of the transgenic fish comprises multiple concatemeric genomically integrated copies of a plasmid comprising an assayable mutation target nucleic acid sequence;

gently disaggregating at least a portion of the fish to yield disaggregated fish material;

digesting the disaggregated fish material with a proteinase for a period of no longer than about 1-½ hours at a temperature of about 37° C.;

extracting DNA comprising the mutation target nucleic acid sequence from the disaggregated fish material sufficient to detect a mutation in the mutation target nucleic acid sequence; and

detecting the presence of a mutation in the mutation target nucleic acid sequence.

2. The method of claim **1** wherein the extracting step yields extracted DNA comprising plasmid DNA and chromosomal DNA.

3. The method of claim **2** further comprising cleaving the extracted DNA with a restriction endonuclease to yield at least one DNA fragment comprising the plasmid-derived mutation target nucleic acid sequence and a multiplicity of DNA fragments comprising chromosomal DNA.

4. The method of claim **3** further comprising separating the DNA fragment comprising the plasmid-derived mutation target nucleic acid sequence from the multiplicity of DNA fragments comprising chromosomal DNA to yield isolated plasmid-derived DNA comprising the mutation target nucleic acid sequence.

5. The method of claim **4** wherein the assayable mutation target nucleic acid sequence comprises a lacZ gene comprising a lacZ operator.

6. The method of claim **5** wherein the step of separating the DNA fragment comprising the plasmid-derived mutation target nucleic acid sequence from the multiplicity of DNA fragments comprising chromosomal DNA comprises:

contacting the DNA fragments with an affinity support comprising a lacZ operator binding material to bind the DNA fragment comprising the plasmid-derived mutation target nucleic acid sequence; and

eluting the bound DNA fragment from the support.

7. The method of claim **6** wherein the contacting step is performed after the cleaving step.

8. The method of claim **1** wherein the providing step comprises providing a transgenic fish that has been or is suspected of having been exposed to a mutagen.

9. The method of claim **6** wherein the contacting step and the cleaving step are performed simultaneously.

10. The method of claim **1** further comprising exposing the transgenic fish to a mutagen prior to extracting the DNA comprising the mutation target nucleic acid sequence.

11. The method of claim **10** wherein the mutagen is selected from the group consisting of a chemical, a radioisotope and electromagnetic radiation.

12. The method of claim **1** wherein the DNA is extracted from an organ or tissue of the transgenic fish.

13. The method of claim **12** further comprising analyzing the mutation.

14. The method of claim **13** wherein the step of analyzing the mutation comprises determining a tissue-specific or organ specific mutation frequency.

15. The method of claim **1** wherein the fish is selected from the group consisting of a medaka and a fundulus.

16. The method of claim **1** wherein the assayable mutation target nucleic acid sequence comprises a lacZ gene comprising a lacZ operator.

17. The method of claim **16** wherein the step of detecting the presence of a mutation in the mutation target nucleic acid sequence comprises:

transforming a host restriction-negative, lacZ⁻galE⁻ bacterial host with the recovered DNA comprising the lacZ gene;

culturing the transformed bacterial host on a lactose-containing or lactose analogue-containing medium; and

selectively detecting a transformed bacterial host that comprises a mutation in the lacZ gene, wherein growth of the bacterial host is indicative of the existence of said mutation.

18. The method of claim **17** comprising, prior to transforming the bacterial host, ligating the recovered DNA to yield a circular DNA.

19. The method of claim **1** wherein the assayable mutation target nucleic acid sequence comprises a nucleic acid sequence selected from the group consisting of a lacI gene sequence, a lacZ gene sequence and a lac promoter sequence.

20. The method of claim **1** wherein the step of detecting the presence of a mutation in the mutation target nucleic acid sequence comprises performing a bioassay.

21. The method of claim **1** further comprising analyzing the mutation in the mutation target nucleic acid sequence.

22. The method of claim **21** wherein the step of analyzing the mutation comprises determining the nucleic acid sequence of at least a portion of the mutation target nucleic acid sequence.

23. A method for evaluating the mutagenicity of a suspected mutagen comprising:

exposing a transgenic fish to a suspected mutagen, wherein the DNA of the transgenic fish comprises multiple concatemeric genomically integrate copies of a plasmid comprising an assayable mutation target nucleic acid sequence;

gently disaggregating at least a portion of the fish to yield disaggregated fish material;

digesting the disaggregated fish material with a proteinase for a period of no longer than about 1-½ hours at a temperature of about 37° C.;

extracting DNA comprising the mutation target nucleic acid sequence from the disaggregated fish material sufficient to detect a mutation in the mutation target nucleic acid sequence; and

detecting the presence of a mutation in the mutation target nucleic acid sequence.

24. The method of wherein **23** wherein the DNA is extracted from an organ or tissue of the transgenic fish.

25. The method of claim **24** furthers comprising analyzing the mutation.

26. The method of claim **25** wherein the step of analyzing the mutation comprises determining a tissue-specific or organ specific mutation frequency.

27. The method of claim **23** wherein the fish is selected from the group consisting of a medaka and a fundulus.

28. The method of claim **23** wherein the assayable mutation target nucleic acid sequence comprises a nucleic acid sequence selected from the group consisting of a lacI gene sequence, a lacZ gene sequence and a lac promoter sequence.

29. The method of claim **23** further comprising analyzing the mutation in the mutation target nucleic acid sequence.

30. The method of claim **29** wherein the step of analyzing the mutation comprises determining the nucleic acid sequence of at least a portion of the mutation target nucleic acid sequence.

31. The method of claim **29** wherein analyzing the mutation comprises determining a mutation spectrum of the suspected mutagen.

32. The method of claim **23** wherein the assayable mutation target nucleic acid sequence comprises a lacZ gene comprising a lacZ operator.

* * * * *

EXHIBIT 4

Molecular Marine Biology and Biotechnology (1993) 2(3) 181–188

Efficiency of introns from various origins in fish cells

O. Hernandez Bétancourt,† J. Attal, M.C. Théron, C. Puissant, and L.M. Houdebine*
Unité de Différenciation Cellulaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France

M. Bearzotti
Unité de virus des Poissons, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cedex, France

Abstract

Several vectors containing (1) regulatory regions from Rous sarcoma virus (RSV), human cytomegalovirus (CMV), and herpes simplex thymidine kinase (TK); (2) introns from early or late SV40 genes and from trout growth hormone gene (tGH); (3) chloramphenicol acetyltransferase gene (CAT); and (4) transcription terminators from SV40 were transfected into carp EPC cells, salmon CHSE cells, tilapia TO_2 cells, quail QT6 cells, and hamster CHO cells. CAT activity was measured in extracts from several cell lines 3 days after transfection and in the fish EPC stable clones. The CMV and RSV promoters were the most potent in all cell types. The intron from late SV40 genes (VP_1 intron) worked properly in QT6 and CHO cells but not in EPC and very weakly in TO_2 cells. The tGH intron was efficient in all cell types but preferentially in fish cells. The small t intron from SV40 was processed in all cell types. The small t and, to a lesser extent, the tGH introns amplified expression of *cat* gene in stable clones, in comparison to the transiently transfected cells. These results indicate that elements from mammalian genes may not be properly recognized by the fish cellular machinery and in an unpredictable manner. This finding suggests that vectors prepared to express foreign genes in transfected cultured fish cells and transgenic fish should preferably contain DNA sequences from fish genes or, alternatively, those sequences from mammalian genes that have been previously proved to be compatible with the fish cellular machinery.

*Correspondence should be sent to this author.
†Permanent address: Centro de Ingeniera Genetica y Biotechnologia, P.O. Box 6162, La Habana, Cuba.

Introduction

Gene transfer into cultured fish cells and into fish embryos has led to limited success in the past years due to the frequent low efficiency of the vectors used. Transfection of foreign genes into fish cells and DNA microinjection into early embryos to obtain transgenic fish, however, proved to raise no particular problems (Houdebine and Chourrout, 1991). In most cases, investigators used gene constructs prepared to work in mammalian cells. Recent studies have shown that several promoters from higher vertebrates can drive transcription of foreign genes with good efficiency in fish cells (Foster et al., 1987; Gedamu et al., 1990; Inoue et al., 1990; Liu et al., 1990; Friedenreich and Schartl, 1990; Bearzotti et al., 1992). In a recent work, we observed that, although the Rous Sarcoma virus (RSV) promoter was a potent regulatory element to direct the expression of *cat* gene followed by the small t SV40 intron and the SV40 early genes terminator, it was only very poorly able to express bovine growth hormone (GH) complementary DNA (cDNA) followed by the GH gene terminator, and totally unable to express the complete bovine GH gene (Bearzotti et al., 1992). These results raised several questions, particularly regarding whether introns and terminators from mammalian genes are always efficiently recognized by the fish cellular machinery.

The experiments described herein were undertaken to clarify these points. For that purpose, a family of vectors were constructed. In all cases, the *cat* gene was used as the reporter gene. The promoters used were essentially those from RSV and human cyto megalovirus (CMV). The efficiency of several introns was compared: the small t intron

from early SV40 genes, the VP_1 intron from late SV40 genes, and the first intron from the trout GH gene. In all cases (except for the previously constructed pRSV chloramphenicol acetyltransferase [CAT] and pCMV CAT, which contain the early gene SV40 transcription terminator), the late gene SV40 transcription terminator, which is reputed to be highly efficient (Carswell and Alwine, 1989), was used. Promoters per se and more generally gene constructs show different potency and in a somewhat unpredictable manner, according to the cell type in which they are transfected. Several fish cell lines from different tissues and from different species were therefore used to tentatively establish a general rule on the efficiency of the different elements of the constructs and particularly of introns.

To define a possible frontier between higher and lower vertebrates for the efficiency of the constructs, the plasmids were transfected not only in fish cell lines but also in a bird cell line (QT6) and in a mammalian cell line (CHO). Experiments carried out in transfected mammalian cells have shown several times that introns may have no significant stimulatory and even in some cases an inhibitory effect in transient assay. On the contrary, introns seem more important or even essential for stably integrated genes in cultured cells and in transgenic animals (Palmiter et al., 1991). The different constructs were therefore systematically assayed in transiently transfected cells and in stable clones. The data reported herein indicate that fish cells show some preference for the fish intron but that elements from mammalian genes can most likely be used successfully on condition to have been tested case by case in fish cells. This work is a step toward construction of efficient vectors for transgenesis in fish.

Results

Transient expression of cat gene

When transferred into EPC cells, pCMV *cat* and pRSV *cat* showed high efficiency, a result that is in agreement with our previous work (Bearzotti et al., 1992). CMV enhancer was much less efficient when associated with tk promoter (Figure 1A). The three plasmids containing the VP_1 intron were totally inactive in EPC cells, and withdrawal of this intron allowed *cat* gene to be expressed, although to a limited extent. The intron from trout GH gene was active only when RSV promoter was used. Unexpectedly, even the $pS_2C\ \Delta\ VP_1$ *cat* and pS_2R INTT *cat* were poorly active, in comparison to pRSV *cat*.

In the salmon CHSE cells, pCMV *cat*, pCMVTK *cat*, and pRSV *cat* were also active, whereas the plasmids containing the VP_1 intron were totally inefficient. Withdrawal of the VP_1 intron restored the activity of these plasmids. The intron from trout GH gene showed moderate or no stimulatory action (Figure 1B). All the CAT activities were much lower in CHSE cells than in other cell lines, which most likely reflects a lower transfection efficiency.

Tilapia TO_2 cells were cultured at 30°C during the growth phase and maintained at 20° or 37°C after transfection. The relative *cat* gene expression obtained with the different vectors was essentially similar at low and high temperature. All the CAT activities were 50 to 100 times lower at 20°C (Figure 1C, D). pCMVTK *cat*, and pRSV *cat* were active in TO_2 cells; pCMV *cat* was the most potent. The presence of the VP_1 intron reduced considerably, although not completely, the capacity of the various promoters to direct *cat* gene expression. As in the EPC cells, the intron from trout GH gene allowed a better expression of *cat* gene than the VP_1 intron.

In contrast to fish cells, quail QT6 and hamster CHO cells readily expressed *cat* gene when the VP_1 intron was present in the constructs. Withdrawal of this intron even reduced the level of CAT activity. The intron from trout GH gene inhibited more or less *cat* gene expression in QT6 cells (Figure 1E), and more markedly in CHO cells (Figure 1F).

cat Gene expression in stable EPC clones

To evaluate the possible role of introns when foreign genes are integrated in cultured cells or possibly in transgenic fish, stable EPC clones harboring the various gene constructs were obtained. The most active plasmids in the transient transfection assays were essentially those also showing the highest activity in the stable clones. Unexpectedly, however, pCMV *cat* and pRSV *cat* were extremely potent in the stable EPC clones (Figure 2A).

An examination of the CAT messenger RNA (mRNA) size using the Northern blotting technique revealed that the intron from trout GH gene was correctly processed in EPC cells, because the size of the mRNA was similar to that of the mRNA resulting from constructs having no intron (Figure 2B). As expected, the size of the CAT mRNA was slightly larger with pCMV *cat* and pRSV *cat*, because the 3' OH untranslated region after the CAT cDNA was longer in these gene constructs

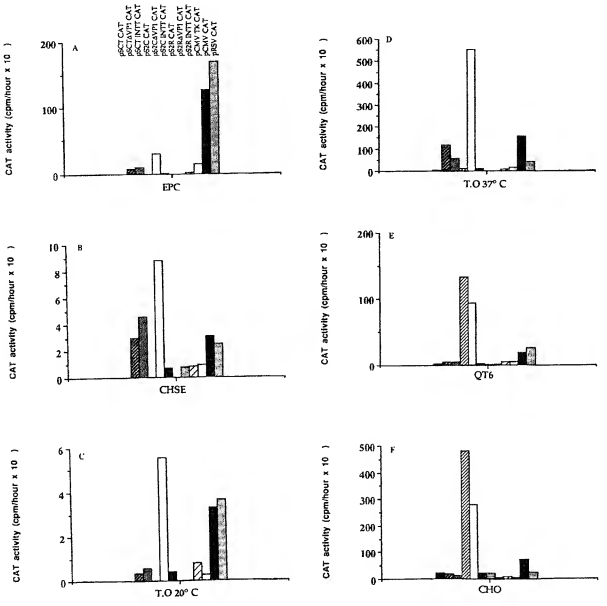


Figure 1. Transient cat gene expression in the various cell lines. Extracts were obtained after repeated freezing and thawing of trypsinized cells. Results are the means of duplicates from two independent dishes after subtraction of the blanks. They are expressed as cpm of (^{3}H)acetylated chloramphenicol per hour and per 10 μg proteins. (A) EPC. (B) CHSE. (C) TO_3(20°C). (D) TO_4(37°C). (E) QT6. (F) CHO.

than in all the plasmids deriving from pSV1. With the pS_2C cat construct, a slight mRNA signal was visible in the upper part of the gel, suggesting that the mRNA has been synthesized to some extent but not properly processed.

In the pCMV cat and pRSV cat, the SV40 small t intron is very small (66 bp). Examination of the Northern blot (see Figure 2B) cannot reveal if the intron sequence was still present in the mRNA. To visualize this intron sequence, Northern blots were hybridized independently with the CAT cDNA probe and with an oligonucleotide synthetic probe

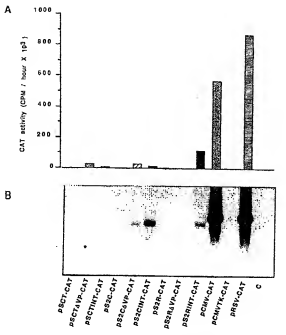


Figure 2. The cat gene expression in stable EPC clones. Transfections were carried out for each plasmid in two independent dishes. After selection with G418, the clones were amplified without being separated. Cells from a given dish were used for the CAT assay, and cells from the duplicate dish were used to prepare RNA. Total RNA were extracted using the method described by Puissant and Houdebine (1990). The CAT assays were carried out as for transient expression; 1 μg of proteins were used for pCMV CAT and pRSV CAT, and 10 μg were used for the other plasmids. Ten μg total RNA were separated in agarose gel in the presence of formaldehyde. RNA was transferred on Z-probe membrane and hybridized with labeled CAT cDNA probe (10^8 cpm/μg DNA) using 10^6 cpm/mL hybridization mixture. Conditions were detailed in a previous work Puissant and Houdebine 1990). (A) CAT activity. (B) Visualization of CAT mRNA. (C) EPC untransfected cells.

containing part of the SV40 small t intron. In Figure 3A, it can be seen that in CHO cells, pCMV *cot*, pS_2C *cat*, pS_2C Δ VP_1 *cat*, and pS_2C INTT cot generated mature CAT mRNA of expected sizes. In agreement with previous work that showed the small t intron uses cryptic splice sites in the upstream of the cot gene (Huang and Gorman, 1990), several RNAs smaller than the expected CAT mRNA were generated in CHO, as in EPC cells when pCMV cot was used for transfection. Unexpectedly, the pS_2C INTT cot gave rise to the generation of two bands hybridizing with the cot probe, suggesting that in CHO cells the t GH intron was not quantitatively processed. The same RNA fractions hybridized to an oligonucleotide probe containing a small t intron sequence showed no signal, suggesting that in EPC as in CHO cells, the small t intron was correctly processed and eliminated (Figure 3B). The capacity of the labeled oligonucleotide probe to hybridize to the small t intron sequence was checked using pCMV *cat* cleaved by *Hin*dIII and *Nco*I in a Southern blotting experiment (Figure 3C).

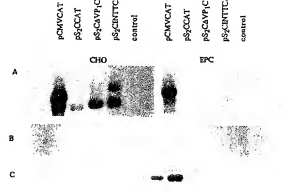


Figure 3. Detection of CAT mRNA and SV40 small t intron sequences in the mRNAs generated by various gene constructs. (A) RNA from stable CHO and EPC clones harboring pCMV CAT, pS_2C CAT, pS_2C Δ VP, CAT, and pS_2R INTT CAT was hybridized with the CAT cDNA probe in conditions described in Figure 3. Control is untransfected cells. (B) The same RNA fractions were hybridized with a labeled oligonucleotide containing part of the small t intron sequence (ACACAAACAATTAGAA TCAG). The oligonucleotide was end-labeled with [α-^{32}P]-dATP and T4 kinase. Hybridization was carried out in conditions similar to those described in Figure 3, except for temperature, which was 54°C. After overnight hybridization, the Z-probe membrane was washed for 30 minutes at room temperature in 4 × SSC. Autoradiography was developed after 6 days at −80°. (C) pCMV CAT was cleaved by *Hin*dIII and *Nco*I and subjected to agarose gel electrophoresis and Southern blotting on Z-probe. The expected three bands were visible in UV light in the presence of ethidium bromide. Only the largest containing the small t intron hybridized with the oligonucleotide probe. Autoradiography was carried out overnight. The hybridized bands correspond, respectively, to 20, 50, 100, and 200 ng digested plasmid.

Discussion

The experiments described herein indicate that CMV and RSV promoters are very active in all fish cell types, a fact already observed with mammalian cells (Gorman et al., 1982; Furth et al., 1991). The tk gene promoter reduced the potency of the CMV enhancer in all cell types studied herein, suggesting that this DNA sequence combination should not be used if a high level of expression is wanted. Unexpectedly, the SV40 promoter added upstream of the RSV LTR considerably reduced the potency of the RSV promoter in the fish cells in QT6. An almost similar combination of promoters injected into early zebrafish and medaka embryos was able to drive the expression of the *cat* gene (Stuart et al., 1990; Chong and Vielkind, 1989). The SV40 early gene promoter was shown to be very weak in EPC cells (Bearzotti et al., 1992) and in transgenic trouts harboring a construct containing the human GH cDNA and an intron from human α-globin gene (Guyomard et al., 1989). This viral sequence may exert some negative effect in fish cells when associated to some other regulatory elements. This putative negative effect was not visible with the CMV regulatory sequence, which remained highly active when pS_2C *cat* or $pS_2C\ \Delta\ VP_1$ *cat* was transfected in all cell types.

pS_2C *cat* was particularly potent in CHO cells. This potency may be due to several factors: (1) the SV40-CMV combination may generate a very potent regulatory element; (2) the SV40 small t intron has been shown not to be an optimal intron because it does not contain the minimal length to be efficiently processed (Fu et al., 1988) and it may utilize cryptic upstream splicing sites in the *cat* gene (Huang and Gorman, 1990) (the VP_1 intron may be more efficient than the small t intron in mammalian cells); and (3) the SV40 late genes terminator has been shown to be 5 times more potent that its early gene counterpart in mammalian cells (Carswell and Alwine, 1989). All these factors may account for the fact that pS_2C *cat* is a more potent vector than pCMV *cat* in CHO cells in the transient assay. The former may be useful when a high level of gene expression is desired in a mammalian cell.

The results reported herein show with no ambiguity that the VP_1 intron is a strong inhibitor of gene expression in fish cells when associated with the *cat* gene. However, this effect is not an all or none phenomenon, because TO_2 cells expressed slightly but significantly the *cat* gene with pS_2C *cat*, at least at 37°C. The VP_1 intron normally works in mammalian cells maintained at 37°C. The poor efficiency of the VP_1 intron in EPC cells cannot be ascribed to temperature because pS_2C *cat* remained poorly active in the TO_2 cells, even at 37°C. In contrast, experiments in progress in our laboratory indicate that the VP_1 intron is significantly active in EPC cells when associated to bovine or tilapia GH cDNA. It is not known if the VP_1 intron prevents the transcriptional step in fish cells or if it reduces the stability of the pre-mRNA, which are not processed efficiently. Whatever the process, this observation is in agreement with a previous work in which we showed that bGH cDNA but not bGH gene can be expressed in EPC cells (Bearzotti et al., 1992). Human GH gene introns have also been shown not to be fully processed in EPC cells (Friedenreich and Schartl, 1990), and introns from mammalian genes are not readily eliminated in cells of another lower vertebrate, Xenopus oocytes (Michaeli et al., 1988).

As opposed to the VP_1 intron, the SV40 small t intron was fully processed in fish and mammalian cells, which gives additional support to the idea that the elimination of a given intron may depend on the genes to which it is associated and in which cell type it is processed. In this respect, it is striking that the SV40 small t intron is a better stimulator of gene expression in transegnic mice than other introns when present in the upstream of a cDNA (Palmiter et al., 1991). The fish cells may preferably process introns from fish genes in some cases, but it seems impossible to consider that an intron from a fish gene will be systematically more potent than an intron from a mammalian gene in a fish cell. The intron to be inserted in a gene construct should ideally be chosen case by case according to the problem under study. The comparison between CAT activity in transiently transfected cells and in the EPC stable clones showed that the 2 introns functional in the fish cells (the small t and the tGH introns) favored *cat* gene expression when the foreign DNA was integrated. This finding suggests that a functional intron may be essential to obtain high transgene expression in fish.

The level of expression was unexpectedly high in the stable clones of EPC and CHO cells harboring pCMV *cat* and pRSV *cat*, in comparison to transiently transfected cells. It is hard to believe that the small t intron is the only factor that amplified expression of the integrated DNA. Preliminary experiments carried out in our laboratory revealed that the small t intron not surrounded by other sequences from the SV40 genome is only moderately efficient in transient or stable expression. The pCMV *cat* and pRSV *cat* contain both in their 3′ OH

end a matrix-attached region (MAR) from the SV40 genome (Pommier et al., 1991). This kind of sequence has been shown to greatly enhance expression of foreign genes when they are integrated in the genome of cultured cells or of transgenic animals (Bonifer et al., 1991). The high expression of integrated pRSV *cat* and pCMV *cat* in EPC cells might be explained by the presence of the SV40 MAR sequence. More generally, the possible stimulatory effect of the SV40 MAR sequence in the numerous vectors containing the SV40 small t intron and the polyadenylation signal may have been underestimated. If so, addition of the MAR from SV40 to constructs to be expressed in fish cells and in transgenic fish should greatly favor their expression. Experiments have been undertaken to verify if this hypothesis is correct.

Materials and Methods

Cell culture and transfection

Cells were transfected using lipofectin (BRL). The plasmids containing the *cat* gene (10 μg) were mixed with lipofectin (10 μL), and the mixture was added to 10^7 cells. After 3 days, cells were collected to evaluate CAT activity. Stable EPC clones were obtained by adding pRSVneo (2 μg) to the constructs containing the *cat* gene and using G-418 (500 μg/mL medium) for selection.

Chloramphenicol acetyl transferase assay

The CAT assay was carried out essentially according to the method described by Nordeen and associates (1987) with the following modifications. This assay is based on the use of (^3H) sodium acetate as precursor, it is very simple and cheap. The amount of labeled sodium acetate required for the assays was evaporated to dryness under vacuum to reduce blanks. The test was performed in 2-mL Eppendorf tubes. The reaction mixture contained 100 mmol/L Tris (pH, 7.8), 6 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.5 mmol/L coenzyme A, 37 mmol/L ATP, 7.5 μU acetyl CoA synthetase, 1 mmol/L chloramphenicol, and 4 μCi (^3H) sodium acetate (2.5 Ci/mmol; Amersham) in a final volume of 100 μL. Details to prepare and store these solutions were described by Nordeen and colleagues (1987). Various amounts of cell extracts (5–50 μL) were added to each tube. Incubations were carried out at 37°C for periods of 1 to 24 hours. The reaction was stopped by adding 0.4 mL 7 mol/L urea in water. One mL scintillation mixture containing PPO (2,5 diphenyloxazole) and POPOP (1,4-bis[5-phenyl-2-oxazolyl]-benzene) was added to each tube.

After a brief vortexing, which favors the trapping of (^3H) acetylated chloramphenicol in the organic phase, the tubes were directly counted in a scintillation counter. This test allowed detection of CAT activity as low as 0.0005 U. It showed good linearity (up to 250,000 cpm of (^3H) acetylated chloramphenicol). The amounts of cell extract and time of incubation were chosen to remain within the linearity of the test. The blanks were of 2,000 to 5,000 cpm and remained constant throughout incubation.

Preparation of vectors

pCMV *cat* and pRSV *cat*, which contain the SV40 small t intron and the early genes terminator, have already been described (Gorman et al., 1982; Foecking and Hofstetter, 1986). All the other gene constructs derived from pSVL (Amersham) are described in Figure 4. This vector contains SV40 late gene promoter, the VP_1 intron from SV40 genes, and the SV40 late genes terminator. In all cases, *cat* gene was inserted into the Xho-Xba_1 sites of the linker. The promoters were in all cases inserted in

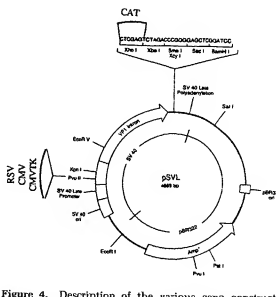


Figure 4. Description of the various gene constructs derived from the pSVL (Amersham). The *cat* gene was introduced in the linker. Additional promoters were inserted into the *Pvu*II site and the trout GH intron in the *Kpn*I/*Xho*I site. Names of the different vectors is given in the text.

the *Pvu*II site with their own tsp. These vectors therefore contained 2 transcription start points: (1) the tsp brought by the foreign promoters, and (2) the remaining SV40 late gene tsp located between the *Kpn*I site and the beginning of the VP_1 intron.

The plasmid pS_2C cat was obtained by inserting the *Xba*I/*Hin*dIII fragment of pCMV cat (Foecking and Hofstetter, 1986) containing the essential CMV regulatory region. The plasmid pSCT cat was obtained by inserting the *Xba*I/*Bg*lII fragment from pCMV TK cat (Boshart et al., 1985) containing the CMV enhancer and the tk gene promoter. The plasmid pS_2R cat was obtained by inserting the *Nde*I/*Hin*dIII fragment of pRSV cat (Gorman et al., 1982) containing the whole RSV LTR. The VP_1 intron was withdrawn by removing the *Kpn*I/*Xho*I fragment of the vectors, which generated $pS_2C\ \Delta$ VP$_1$ *cat*, pSCT Δ VP$_1$ cat, and $pS_2R\ \Delta$ VP$_1$ cat.

The first intron from trout GH gene (Agellon et al., 1988) was inserted into the *Kpn*I/*Xho*I site after removing the VP_1 intron. The fish intron was obtained after PCR amplification of the whole trout DNA using 5′–GTGAGGTACCCATCCTTGGCAAT AAGAGAAAAAAACGGGAC–3′ and 5′–TGCTCG AGATGTTGAAGAGCCGTTGGTTTTCCATCGCC GC–3′ as primers. These oligonucleotides created KpnI and XhoI restriction sites, respectively (the underlined sequences), which generated pS_2C IN'I'T *cat*, pSCT INTT cat, and pS_2R INTT cat.

Acknowledgments

This work was supported by the financial help of the BRIDGE programme of the European Community and by the AIP Transgene of the Institut National de la Recherche Agronomique. The O. Hernandez-Betancourt fellowship was supported by the Centre International des Etudiants Stagiaires. The authors wish to thank D. Chourrout for his constant interest in this work.

References

Agellon, L.B., Davies, S.L., Chen, T.T., and Powers, D.A. (1988). Structure of a fish (rainbow trout) growth hormone gene and its evolutionary implications. Proc *Natl Acad Sci USA* 85:5136–5140.

Bearzotti, M., Perrot, E., Michard-Vanhee, C., Jolivet, G., Attal, J., Theron, M.C., Puissant, C., Dreano, M., Kopchick, J.J., Powell, R., Gannon, F., Houdebine, L.M., and Chourrout, D. (1992). Gene expression following transfection of fish cells. *J Biotechnol* 26:315–325.

Bonifer, C., Hecht, A., Saueressig, H., Winter, D.M., Sipper, A.E., (1991). Dynamic chromatin: the regulatory domain organization of eukaryotic gene loci. *J Cel Biochem* 47:99–108.

Boshart, M., Weber, F., Jahn, G., Dorsch-Häsler, K., Fleckenstein, B., and Schaffner, W. (1985). A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* 41:521–530.

Carswell, S., and Alwine, J.C. (1989). Efficiency of utilization of the simian virus 40 late polyadenylation site: effects of upstream sequences. *Mol Cell Biol* 9:4248–4258.

Chong, S.S.C., and Vielkind, J.R. (1989). Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes*) eggs in the form of plasmid DNA, recombinant phage particles and its DNA. *Theor Appl Genet* 78:369–380.

Foecking, M.K., and Hofstetter, H. (1986). Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene 45:101–105.

Foster, R., Olsson, P.E., and Gedamu, L. (1989). Calcium phosphate-mediated transfection alters metallothionein gene expression in response to Cd^{2+} and Zn^{2+}. *Mol Cell Biol* 9:4105–4108.

Friedenreich, H., and Schartl, M. (1990). Transient expression directed by homologous and heterologous promoter and enhancer sequences in fish cells. *Nucleic Acids Res* 18:3299–3305.

Fu, X.Y., Colgan, J.D., and Manley, J.L. (1988). Multiple cis-acting sequence elements are required for efficient splicing of simian virus 40 small-t antigen pre-mRNA *Mol Cell Biol* 8:3582–3590.

Furth, P.A., Hennighausen, L., Baker, C., Beatty, B., and Woychick, R. (1991). The variability in activity of the universally expressed human cytomegalovirus immediate early gene 1 enhancer/promoter in transgenic mice. Nucleic Acids Res 19:6205–6208.

Gedamu, L., Olsson, P.E., and Zafarullah, M. (1990). Regulation of rainbow trout metallothionein genes. In: *Transgenic Models in Medicine and Agriculture*. Church, R. (ed.). New York: Willey-Liss, pp. 101–108.

Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastan, I., and Howard, B.H. (1982). The rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc *Natl Acad Sci USA* 79:6777–6781.

Guyomard, R., Chourrout, D., Leroux, C., Houdebine, L.M., and Pourrain, F. (1989). Integration and germ line transmission of foreign genes microinjected into fertilized trout eggs. Biochimie 71:857–883.

Houdebine, L.M., and Chourrout, D. (1991). Transgenic in fish. Experientio 47:891–897.

Huang, M.T.F., and Gorman, C.M. (1990). The simian virus 40 small-t intron, present in many common expression vectors, leads to aberrant splicing. *Mol Cell Biol* 10:1805–1810.

Inoue, K., Yamashita, S., Hata, J.I., Kabeno, S., Asada, S., Nagahisa, E., and Fujita, T. (1990). Electroporation as a new technique for producing transgenic fish. *Cell Diff Dev* 29:123–128.

Lannan, C.N., Winton, J.R., and Fryer, J.L. (1984). Fish cell lines: establishment and characterization of nine cell lines from salmonids. *In Vitro* 20:671–676.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R., and Hackett, P.B. (1990). Development of expression vectors for transgenic fish. *Bio/Technol* 8:1268–1272.

Michaeli, T., Dan, Z.Q., and Prives, C. (1988). An excised SV40 intron accumulates and is stable Xenopus laevis oocytes. Gene Dev 2:1012–1020.

Nordeen, S., Green, P.P., and Fowlkes, D.M. (1987). A rapid sensitive and inexpensive assay for chloramphenicol acetyl transferase. DNA 6:173–178.

Palmiter, R.D., Sandgren, E.P., Avarbock, M.R., Allen, D.D., and Brinster, R.L. (1991). Heterologous introns can enhance expression of transgenes in mice. Proc Natl Acad Sci USA 88:478–482.

Pommier, Y., Capranico, G., Orr, A., and Kohn, K.W. (1991). Distribution of topoisomerase II cleavage sites in simian virus 40 DNA and the effects of drugs. J Mol Biol 222:909–924.

Puissant, C., and Houdebine, L.M. (1990). An improvement of the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Biotechniques 8:148–150.

Stuart, G.W., Vielkind, J.R., McMurray, J.V., and Westerfield, M. (1990). Stable lines of transgenic zebrafish exhibit reproductible patterns of transgene expression. Development 109:577–584.

EXHIBIT 5

Journal of Biotechnology, 26 (1992) 315–325

BIOTEC 00795

Gene expression following transfection of fish cells

Monique Bearzotti [a], Elisabeth Perrot [b], Christine Michard-Vanhee [b], Geneviève Jolivet [c], Joé Attal [c], Marie-Claire Theron [c], Claudine Puissant [c], Michel Dreano [d], J.J. Kopchick [e], Richard Powell [f], Franck Gannon [f], Louis-Marie Houdebine [c] and Daniel Chourrout [b]

[a] *Unité des Virus de Poissons,* [b] *Laboratoire de Génétique des Poissons, and* [c] *Unité de Différenciation Cellulaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cédex, France*
[d] *ARES-SERONO, Genève, Switzerland*
[e] *Department of Zoology and Biomedical Sciences, Ohio University, Athens, OH, 45701, USA*
[f] *National Diagnostic Center, University College, Galway, Ireland*

(Received 10 October 1991; revision accepted 12 March 1992)

Summary

Various genes containing different transcriptional regulatory elements (TRE) and the bacterial marker gene coding for chloramphenicol acetyl transferase were transfected into several fish cell lines to evaluate the efficiency of expression in comparison with mammalian cells. The CMV and RSV TRE were the most efficient non-inducible promoters in directing reporter gene expression. RSV and CMV appeared of similar potency in a stable fish cell line. The human HSP-70 promoter showed high potency in a carp and in a trout cell line after thermal induction. This promoter also induced the synthesis of human growth hormone directed by the corresponding cDNA, but not by the gene. RSV TRE was also able to drive the synthesis of bovine growth hormone when attached directly to the cDNA but not to the gene. These data suggest that non-fish gene TRE can be used to express foreign genes in fish cells or transgenic fish; however, in most cases they are relatively inefficient. The data also suggest that the translation and secretion machinery of fish cells can express efficiently foreign genes but that mammalian introns might be not processed properly in some cases.

Gene constructs; Transfection; Fish cells; CAT assay

Correspondence to: L.M. Houdebine, Unité de Différenciation Cellulaire, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas Cédex, France.

Introduction

Experiments carried out over the last 6 years in several laboratories have shown that transgenesis in fish is relatively easy to obtain. In most species studied so far, pronuclei are not visible and microinjection of a high copy number of the genes leads to the generation of mosaic transgenic fish (Chourrout et al., 1990; Houdebine and Chourrout, 1991). Previously, very few fish genes were available for gene transfer to fish and the efficiency of transgenesis was unknown. Initially, most laboratories chose to microinject available gene constructs containing mammalian DNA with strong TRE and expected these genes to be expressed with some efficiency in the transgenic fish. In most cases, expression of transgenes proved to be very low although they were not rearranged and properly transmitted to progeny (Chourrout et al., 1990; Houdebine and Chourrout, 1991). A systematic study of the efficiency of foreign gene expression in fish cells then appeared necessary and it has been undertaken by a few laboratories (Foster et al., 1989; Friedenreich and Schartl, 1990; Inoue et al., 1990; Gedamu et al., 1990). The experiments described in the present paper detail additional information on the efficiency of expression of several mammalian TRE and genes in fish cell lines.

Materials and Methods

Gene constructs

Gene constructs already prepared for work in mammalian cells were used. pSV518 (provided by Dr. J. Lupker) contained the early SV40 promoter, the human growth hormone (hGH) cDNA, a-globin intron and SV40 early terminator. HSP70-CAT which contained the heat shock protein promoter and the chloramphenicol acetyl transferase gene (CAT gene) and HSP70-hGH cDNA and gene were prepared by Dr. M. Dreano (Dreano et al., 1988). pCMVIEbGH (Pasleau et al., 1987) and pCMVbGH (unpublished data) contained the promoter of early gene cytomegalovirus and bovine growth hormone gene. pRSVbGH (pbGH5) and pRSVhGH contained the LTR from Rous Sarcoma Virus (Gorman et al., 1982a, b) and the bovine or human growth hormone gene. pbGH7 contained RSV LTR as TRE, bovine GH cDNA and the terminator of bovine GH gene (Pasleau et al., 1987). pCMV CAT, provided by Dr. H. Hoffstetter (Foecking and Hoffstetter, 1986), contained the early gene TRE of human cytomegalovirus and the CAT gene. pCMVTK CAT (provided by Dr. M. Schartl) contained the enhancer of human cytomegalovirus, the thymidine kinase promoter from Herpes Simplex virus and the CAT gene (Friedenreich and Schartl, 1990). p-Actin CAT (provided by Dr. T. Mohum) contained the Xenopus β-actin promoter fused to CAT gene (Mohum et al., 1987). pSV_2 CAT contained the early SV40 promoter fused to CAT gene (Gorman et al., 1982a). pMT-hGH contained the mouse metallothionein promoter fused to hGH gene (Palmiter et al., 1983). pSVTK CAT (provided by Dr. M. Schartl) contained the enhancer of SV40 early gene and the thymidine kinase gene promoter of Herpes Simplex virus. pRSVneo and pSV_2neo were those previously

described (Gorman et al., 1983). pBSpac (containing the puromycin resistance gene fused to SV40 promoter) was provided by Dr. A. Jimenez (Delaluna et al., 1988). All the plasmids described above were previously used in CHO cells and proved to be highly active.

Cell culture and transfection

Three fish cell lines were used: EPC, derived from carp epidermal herpes virus-induced hyperplastic lesions (Fijan et al., 1983); RTH (Fryer et al., 1981) and RTG (Wolf and Quimby, 1962), two trout cell lines derived from liver and gonads, respectively. They were routinely cultured on plastic dishes at 30°C, 20°C and 20°C, respectively. The culture medium was Eagle medium modified by Stoker for BHK21 cells, buffered by 20 mM Tris-HCl pH 7.4, and containing 10% foetal calf serum, 10% tryptose phosphate broth, 100 U ml^{-1} penicillin and 100 $\mu g\ ml^{-1}$ streptomycin.

About 10^7 cells (80% of confluency) were transfected using DEAE-dextran (200 $\mu g\ ml^{-1}$) for 4 h in the presence of 10 $\mu g\ ml^{-1}$ DNA followed by an osmotic shock with 10% dimethylsulfoxide for 3 min. Alternatively, lipofectin (BRL) (10 $\mu l\ ml^{-1}$) with DNA (10 $\mu g\ ml^{-1}$) were added to the cells for 4 h as recommended by the manufacturer.

When co-transfections were carried out, the plasmids for selection (pRSVneo, pSV_2neo or pBSpac, 2 $\mu g\ ml^{-1}$) were added and the reporter plasmids (8 $\mu g\ ml^{-1}$).

To establish stable cell lines, geneticin (Sigma) 200 $\mu g\ ml^{-1}$ or puromycin (Sigma) 5 $\mu g\ ml^{-1}$ were added 3 d after transfection and maintained for at least 15 d. Individual clones were not isolated but studies were performed on pools of selected clones.

Measurement of gene expression

Chloramphenicol acetyl transferase (CAT) activity was measured by the classical assay using thin layer chromatography (Gorman et al., 1982b). The results are qualitatively expressed by direct inspection of the autoradiograph, or as the percentage of conversion of labelled chloramphenicol into acetylated chloramphenicol. For quantitative estimation of CAT activity, the radioactive spots detected after autoradiography were eluted into liquid scintillation vials for counting of radioactivity. The percentage conversion of labelled chloramphenicol into acetylated forms was calculated per mg of protein in the cell extracts.

Human and bovine GH were measured in the culture medium using specific radioimmunoassay provided by Dr. J. Lupker and NIH, respectively.

Northern blot analysis

Different cell clones from transfected EPC cells harbouring pbGH7, pbGH5, pCMVIEbGH and pCMVbGH were grown to half-confluency. Total RNA was extracted using the isothiocyanate-phenol-chloroform method. Poly A RNA were isolated using poly U-sepharose chromatography. Northern blot analysis was carried out using conventional methods. After electrophoresis, RNA was transferred to Zeta probe nylon membrane in $10 \times$ SSC. RNA was fixed by UV

irradiation. Hybridization was carried out in 500 mM sodium phosphate buffer at pH 7.2, containing 7% SDS in the presence of 1×10^6 cpm ml^{-1} of ^{32}P labelled bGHcDNA (10^8 cpm μg^{-1}) DNA). After an overnight hybridization at 65°C, the filters were washed in 50 mM sodium phosphate buffer at room temperature for 2 h and at 65°C for 15 min. Autoradiography was carried out as stated in the legend of Fig. 4.

Results

Generation of stable cell lines

The three cell lines – EPC, RTH and RTG – were transfected by conventional methods with plasmids capable of providing resistance to antibiotics. DEAE dextran, polybrene and lipofectin proved to be efficient mediators for transfection. For the sake of convenience, lipofectin was used routinely. Several plasmids containing the neo^r gene gave rise to stable EPC clones resistant to geneticin. pRSVneo and pSV_2neo plasmids resulted in the highest number of clones generated. Other plasmids containing the same neo^r gene under the control of the promoter from Herpes thymidine kinase (TK), or mouse leukemia virus LTR, generated less geneticin-resistant clones. Growth of the RTG cell line could not be inhibited by geneticin at concentrations of the antibiotic as high as 1 or 2 mg ml^{-1}. For RTG and RTH cell lines, selection with puromycin was used.

Kinetics of CAT gene expression in transient assay

pCMV CAT was transfected in the three cell lines in the presence of lipofectin. CAT assays were carried out at different periods after transfection in independent dishes. Results shown in Fig. 1 indicate that, of the three cell lines, CAT expression was the highest in EPC 3–6 days post-transfection. In this respect, foreign DNA transfected into fish cells exhibit the same pattern of expression as their mammalian counterparts. In further experiments, unless stated, CAT assays were performed 4 d after transfection.

The CAT gene was also expressed much more efficiently in the EPC cells than in the other two lines. This fact was observed regularly with other plasmids expressing transiently a reporter gene and with plasmids conferring resistance to antibiotics as well, suggesting that EPC cells are much more readily transfected (with the different transfection techniques) than RTG and RTH cells. The values obtained with RTG and RTH were low when expressed as percent conversion per mg protein. About 50 mg of protein from RTH and RTG cell extracts, and 1–10 mg of protein from EPC cell extracts, were used in the assays. The tests were thus carried out in good experimental conditions and the data reported in Fig. 1 really reflect the relative efficiency of transfection and expression for the three cell lines.

Comparison of various promoters fused to CAT gene

Several plasmids were transfected into EPC cells and the CAT activity was measured in cell extracts 3 d post-transfection. All these plasmids were tested

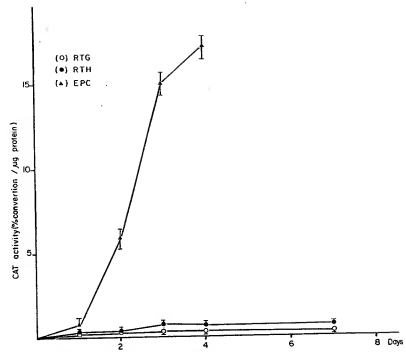


Fig. 1. Kinetics of CAT gene expression. pCMV CAT was transfected into EPC (▲), RTH (●) and RTG (○) using lipofectin. CAT assay was carried out after 1, 2, 3, 4 and 7 d. Results are the means (±SEM) of three independent dishes.

regularly using mammalian cells where they showed good efficiency. pSV_2 CAT was totally inactive in these experimental conditions and p-actin CAT showed low, but detectable, activities, whereas pRSV CAT and pCMV CAT were very potent (Fig. 2). RSV and CMV thus appeared as strong transcriptional promoters for fish cells, at least for the EPC cell lines in transient expression assays.

The SV40 and CMV enhancers associated with TK promoter were compared with the natural complete SV40 and CMV regulatory regions. The addition of TK promoter stimulated strongly the SV40 enhancer but reduced the efficiency of the CMV enhancer (Table 1). In all cases, however, SV40 enhancer exhibited a much lower activity than the CMV enhancer.

Comparison of RSV and CMV promoters in stable clones

pRSV CAT and pCMV CAT were transfected in independent dishes in the presence of pSV_2 neo. Stably transformed EPC clones resistant to geneticin were analysed for CAT activity. Both groups of clones contained a similar number of CAT gene copies as judged by a Southern blot analysis (Fig. 3A). They also expressed the CAT gene at a similar level as judged by a Northern blot analysis (Fig. 3B). A CAT assay revealed that clones harbouring the pRSV CAT construct

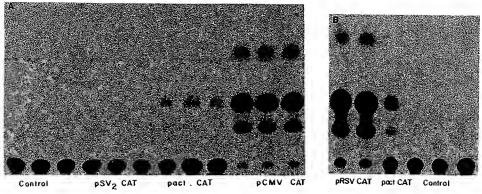


Fig. 2. Expression of CAT gene under the control of various promoters in EPC. The indicated plasmids were transfected by DEAE-dextran in conditions described in Materials and Methods. CAT assays were performed 3 d later, using thin layer chromatography. Each datum results from a culture dish. A and B are two independent experiments.

contained 2.5 times more enzyme than those harbouring the pCMV CAT construct (not shown). Both gene constructs were, therefore, of essentially equivalent potency when expressed transiently or in stable EPC clones. This result is in good agreement with a preliminary experiment that showed, in transgenic trout several months old, pCMV CAT was expressed at a rather high level in the six tissues examined.

Expression of the human HSP70-CAT plasmid

A plasmid containing the CAT gene under the control of human heat shock protein gene TRE and the neo^r gene driven by the SV40 early gene promoter were transfected into EPC and RTH cells. To avoid interference between the transient presence of the plasmids after transfection and the transient stimulation by heat, stable EPC and RTH clones were obtained after selection by geneticin for EPC cells and/or puromycin, respectively. These cells were subsequently exposed to a heat shock (28°C for RTH and 38°C for EPC). In both cases, pools of clones

TABLE 1

Effect of the TK promoter associated with SV40 and CMV enhancers

Plasmids	Protein per assay (mg)	Conversion (%)
pSV_2CAT	100	0
pSV_2TKCAT	100	80
pCMVCAT	1	60
pCMVTKCAT	1	6.5

EPC cells were transfected with lipofectin in conditions depicted in Methods. CAT assays were carried out 3 d later from three and two independent dishes for SV40 and CMV respectively. Results are the means obtained from the three different dishes.

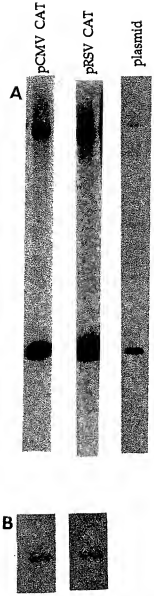


Fig. 3. (A) Southern blot analysis of CAT gene in stable EPC clones. DNA from clones resistant to geneticin was extracted and cleaved by Hind III and Neo I. These restriction enzymes cut pCMV CAT and pRSV CAT at the beginning and in the midst of the CAT gene, respectively. The digested DNA (10 μg) was subjected to Southern blotting and hybridisation was carried out using the labelled CAT gene insert as probe. The plasmid pRSV CAT was used as control. (B) Northern blot analysis of CAT mRNA in stable EPC clones. RNA was extracted and transferred to nylon membranes as described in Materials and Methods. Hybridisation was carried out using the labelled CAT gene insert as probe. Five μg of RNA were used and autoradiography was carried out for 3 d.

TABLE 2

Induction of CAT expression by heat following transfection by HSP-70-CAT

Temperature	Cells	Conversion per mg protein (%)
20°C	RTH	1.5
28°C	RTH	11
30°C	EPC	0
38°C	EPC	19.5

Pools of RTH and EPC cells were heat-stocked at 28°C and 38°C, respectively, for 2 h. CAT assays were carried out after an additional incubation at normal temperature (20°C and 30°C, respectively) for 22 h. Results are expressed as the percentage of labelled chloramphenicol which has become acetylated per mg protein of cell extracts.

(several hundreds for EPC and 30 for RTH) were examined to attenuate variations of expression in individual clones (Table 2). Heat exerted a marked induction of CAT gene expression in both cell types. In comparison to RSV and pCMV TREs, the human HSP70 TRE appears to be quite potent in fish cells.

Expression of GH genes in transfected cells

The different plasmids described in Materials and Methods containing hGH and bGH cDNAs or genes were transfected into EPC cells. The hGH cDNA of pSV518 was not expressed to a significant level in transienty or stably transfected cells. Similarly, the mouse metallothionein-hGH gene was not expressed in the presence of Zn^{2+}. Stable clones harbouring gene constructs containing the neo^r gene under the SV40 promoter control and hGH cDNA or gene fused to the human HSP70 promoter were tested for their capacity to secrete the hormone after a heat shock. The pool of clones (2×10^8 cells per dish) harbouring the hGH cDNA secreted the hormone at a low, but detectable level (1 ng ml^{-1} medium) 10 h after, but not before, the heat shock. The plasmid containing the hGH gene instead of the cDNA remained inactive. This observation is in clear contrast with data obtained with mammalian cells using the same plasmids. Both hGH cDNA and hGH gene were equally effective after the heat shock (Alouani et al., 1992).

Bovine GH gene and cDNA under the control of RSV and CMV promoters were also tested in EPC cells. Pools of clones harbouring pCMV bGH gene, pRSV bGH gene and pRSV bGH cDNA were also examined. Only cells transfected with the pbGH-7 cDNA led to a significant secretion of bGH in the culture medium (Fig. 4). The hormone accumulated in the medium in a linear fashion for 2 weeks.

Northern blots obtained with total RNA or polyA containing RNA from the different pools of bGH clones revealed that a band was visible in fractions extracted from cells harbouring pbGH7. This band was at the position of bGH mRNA extracted from bovine hypophysis. The other clones showed no detectable bGH mRNA but only unspecific hybridization signals also found with RNA from control untransfected EPC cells (Fig. 5).

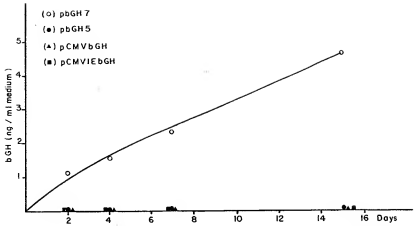


Fig. 4. Expression of bGH in EPC cells. The various plasmids containing bGH gene and cDNA were stably integrated and then activity was evaluated using a radioimmunoassay. The cells were maintained without fetal calf serum during the total period of sampling to avoid a possible interference of serum bovine GH with the radioimmunoassay. About 1×10^5 cells were present in each dish and the medium was not changed over the two weeks of the experiment. (○) pbGH7; (●) pbGH5; (▲) pCMVbGH; (■) pCMVIEbGH.

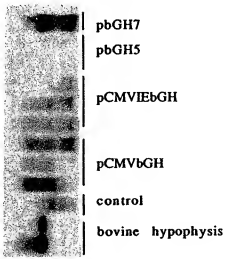


Fig. 5. Northern blot analysis of RNA from transfected EPC cells. Poly A^+ RNA purified from $10A_{260}$ total RNA were added in each well. Total RNA from bovine hypophysis was used as control (1 and 2.5 μg of RNA). Autoradiography was carried out for $4\frac{1}{2}$ h with hypophysis RNA and for 9 d with fish cell RNA.

Discussion

The data reported here indicate that fish cells can be used to define promoter strength to be used for subsequent studies directed at generating transgenic fish. A classification of the various promoters clearly emerges from these experiments. The RSV TRE appears very potent, particularly in transient assay, a fact which is in close agreement with data reported by other groups using the same or different cells and plasmids (Foster et al., 1989; Friedenreich and Schartl, 1990). The CMV TRE also appeared very efficient in fish cells.

The CAT assay is known to be very sensitive, due to amplification of the enzymatic reaction. The measurement of GH in the culture medium gives a different estimation of gene expression efficiency. In this respect, even pbGH7, the most potent in this study, was much less efficient in EPC cells than in CHO cells (not shown). Some of the known strong mammalian TREs may, therefore, be used in fish cells and transgenic fish, but homologous promoters that may prove to be more efficient and appropriate for targeting selectively the expression of the foreign genes in different cell types are still to be found in most cases. The fact that β-actin promoter from carp exhibited a high capacity to drive CAT gene expression in vitro in fish cells and in vivo supports this view (Liu et al., 1990).

The estimation of GH mRNA concentration from Northern blots clearly showed that these molecules did not accumulate readily in the fish cells with the plasmids tested. GH mRNA was found only in cells which express the hormone. This suggests that the translation machinery, including the adaptation of fish tRNA to codons preferentially used for mammalian mRNA and the secretion process, including cleavage of the signal peptide of the preproteins, have not to be considered as strongly limiting, as judged by data reported here. Therefore, it is probably the transcription step, and possibly the processing of the transcripts, including the elimination of introns and stabilization of mRNA in cytoplasm, which are somewhat different in fish and mammalian cells. The use of fish gene or cDNA sequences, instead of their mammalian counterparts, should give additional information on this point.

One point remains striking. The plasmids containing GH genes, rather than the corresponding cDNA, were inactive. This may result from an inefficient processing of the pre-mRNA in fish cells. Such a possibility has been evoked in Xenopus oocytes (Michaeli et al., 1988) and in EPC cells (Friedenreich and Schartl, 1990). Experiments are in progress in our laboratory to evaluate the impact of mammalian and fish introns on expression in mammalian and fish cells.

Acknowledgements

This work was carried out with the financial help of the European Community Biotechnology Action Programme. The authors wish to thank Dr. J. Lupker (Sanofi-Elf Biorecherche, Toulouse, France) for providing pSV518, Dr. M. Schartl (Max Planck Institut für Biochemie, Martinsried, Germany) for providing

pCMVTK-CAT and pSTKCAT, Dr. H. Hoffstetter (Basel) for providing pCMV-CAT, Dr. T. Mohum (Cambridge) for providing p-actin-CAT, and Dr. G. Kann (INRA, Jouy-en-Josas, France) for his help in radioimmunoassays.

References

Alouani, S., l'Hote, D., Marq, J.B., Houdebine, L.M., Montandon, F., Chessebeuf-Padieu, M. and Dreano, M. (1992) Expression of heat shock regulated human growth hormone genes containing or lacking nitrous by NIH-3T3 and fish cell lines. Cell Biol. Toxicol. (in press).

Chourrout, D., Guyomard, R. and Houdebine, L.M. (1990) In: Church, R. (Ed.) Transgenic Models in Medicine and Agriculture. Wiley-Liss, New York, pp. 89–99.

Delaluna, S., Soria, I., Pulido, D., Ortin, J. and Jimenez, A. (1988) Efficient transformation of mammalian cells with constructs containing a puromycin-resistance marker. Gene 62, 121–126.

Dreano, M., Marq, J.B. and Bromley, P. (1988) Antibody formation against heat-induced gene products expressed in animals. Bio/Technology 6, 1340–1342.

Fijan, N., Sulimanovic, D., Bearzotti, M., Muzinic, D., Zwillenberg, L.O., Chilmonczyk, S., Vautherot, J.F. and Dekinkelin, P. (1983) Some properties of the *Epithelioma papulosum cyprini* (EPC) cell line from carp *Cyprinus carpio*. Ann. Virol. Inst. Pasteur 134(E), 207–220.

Foecking, M.K. and Hofstetter, H. (1986) Powerful and versatile enhancer-promoter unit for mammalian expression vectors. Gene 45, 101–105.

Foster, R., Olsson, P.E. and Gedamu, L. (1989) Calcium phosphate-mediated transfection alters metallothionein gene expression in response to Cd^{2+} and Zn^{2+}. Mol. Cell. Biol. 9, 4105–4108.

Friedenreich, H. and Schartl, M. (1990) Transcript expression directed by homologous and heterologous promoter and enhancer sequences in fish cells. Nucleic Acids Res. 18, 3299–3305.

Fryer, J.L., McCain, B.B. and Leong, J.C. (1981) A cell line derived from rainbow trout (*Salmo gairdneri*) Hepatoma. Fish Pathol. 15, 193–200.

Gedamu, L., Olsson, P.-E. and Zafarullah, M. (1990) Regulation of rainbow trout metallothionein genes. In: Church, R. (Ed.), Transgenic Models in Medicine and Agriculture. Wiley-Liss, New York, pp. 101–108.

Gorman, C.M., Merlino, G.T., Willingham, M.C., Pastani, I. and Howard, B.H. (1982a) The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. Proc. Nat. Acad. Sci. USA, 6777–6781.

Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982b) Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. 79, 1044–1051.

Gorman, C.M., Padmanabhan, R. and Howard, B.H. (1983) High efficiency DNA-mediated transformation of primate cells. Science 221, 551–553.

Houdebine, L.M. and Chourrout, D. (1991) Transgenesis in fish. Experientia 47, 891–897.

Inoue, K., Akita, N., Yamashita, S., Shiba, T. and Fujita, T. (1990) Constitutive and inducible expression of a transgene directed by heterologous promoters in a trout liver cell line. Biochim. Biophys. Res. Comm. 173, 1311–1316.

Liu, Z., Moav, B., Faras, A.J., Guise, K.S., Kapuscinski, A.R. and Hackett, P.B. (1990) Development of expression vectors for transgenic fish. Bio/Technology 8, 1268–1272.

Michaeli, T., Pan, Z.Q. and Prives, C. (1988) An excised SV40 intron accumulates and is stable in *Xenopus laevis* oocytes. Genes and Development 2, 1012–1020.

Mohum, T., Garrett, N. and Treisman, R. (1987) *Xenopus* cytoskeletal actin and human c-*fos* gene promoters share a conserved protein-binding site. EMBO J. 6, 667–673.

Palmiter, R.S., Norstedt, G., Gelinas, R.E., Hammer, R.E. and Brinster, R.L. (1983) Metallothionein-human GH fusion genes stimulate growth of mice. Science 222, 809–824.

Pasleau, F., Leung, F. and Kopchick, J.J. (1987) A comparison of bovine growth hormone expression directed by bGH genomic or intronless DNA in transiently transfected eukaryotic cells. Gene 57, 47–52.

Wolf, K. and Quimby, M.C. (1962) Established eurythermic line of fish cells in vitro. Science 135, 1065–1066.

EXHIBIT 6

DEVELOPMENTAL BIOLOGY 192, 289–299 (1997)
ARTICLE NO. DB978779

High-Frequency Generation of Transgenic Zebrafish Which Reliably Express GFP in Whole Muscles or the Whole Body by Using Promoters of Zebrafish Origin

Shin-ichi Higashijima,*,†,[1] Hitoshi Okamoto,‡ Naoto Ueno,† Yoshiki Hotta,§ and Goro Eguchi†,[2]

**PRESTO, Japan Science and Technology Corporation (JST), Japan; †Division of Morphogenesis, National Institute for Basis Biology, Myodaiji-cho, Okazaki, Aichi 444, Japan; ‡Department of Physiology, Keio University, School of Medicine, Shinanomachi, Shinjuku-ku, Tokyo 160, Japan; and §Graduate School of Science, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*

Despite a number of reports on transgenic zebrafish, there have been no reports on transgenic zebrafish in which the gene is under the control of a promoter of zebrafish origin. Neither have there been reports on transgenic zebrafish in which the gene is under the control of a tissue-specific promoter/enhancer. To investigate whether it is possible to generate transgenic zebrafish which reliably express a reporter gene in specific tissues, we have isolated a zebrafish muscle-specific *actin* (*α-actin*) promoter and generated transgenic zebrafish in which the green fluorescent protein (GFP) reporter gene was driven by this promoter. In total, 41 GFP-expressing transgenic lines were generated with a frequency of as high as 21% (41 of 194), and GFP was specifically expressed throughout muscle cells in virtually all of the lines (40 of 41). Nonexpressing transgenic lines were rare. This demonstrates that a tissue-specific promoter can reliably drive reporter gene expression in transgenic zebrafish in a manner identical to the control of the endogeneous expression of the gene. Levels of GFP expression varied greatly from line to line; i.e., fluorescence was very weak in some lines, while it was extremely high in others. We also isolated a zebrafish cytoskeletal *β-actin* promoter and generated transgenic zebrafish using a β-actin–GFP construct. In all of the four lines generated, GFP was expressed throughout the body like the *β-actin* gene, demonstrating that consistent expression could also be achieved in this case. In the present study, we also examined the effects of factors which potentially affect the transgenic frequency or expression levels. The following results were obtained: (i) expression levels of GFP in the injected embryo were not strongly correlated to transgenic frequency; (ii) the effect of the NLS peptide (SV40 T antigen nuclear localization sequence), which has been suggested to facilitate the transfer of a transgene into embryonic nuclei, remained to be elusive; (iii) a plasmid vector sequence placed upstream of the construct might reduce the expression levels of the reporter gene. © 1997 Academic Press

INTRODUCTION

Zebrafish is an excellent model organism for the study of vertebrate development (Kimmel, 1989; Nüsslein-Volhard, 1994). The embryos develop outside the mother and are optically transparent, allowing direct observation of their embryonic development. A relatively short generation time of 2–3 months makes classical genetics feasible. Recent large-scale mutagenesis screening has led to the isolation of a great number of lethal mutations of genes essential for embryonic development (Haffter *et al.*, 1996; Driever *et al.*, 1996).

An important technology in model organisms is the capability of producing transgenic organisms as has been shown for *Drosophila* or mice. Transgenic zebrafish, using several DNA constructs, have been generated in the past 9 years.

[1] To whom correspondence should be addressed at National Institute for Basic Biology, Myodaiji-cho, Okazaki, Aichi 444, Japan. Fax: +81-564-55-7571. E-mail: shinichi@nibb.ac.jp.

[2] Present address: Kumamoto University, Kurokami, Kumamoto 860, Japan.

0012-1606/97 $25.00

Stuart *et al.* (1988, 1990) first showed that DNA injected into the cytoplasm of fertilized zebrafish eggs could integrate into the fish genome and be inherited in the germline. Culp *et al.* (1991) demonstrated that the frequency of germline transmission of a microinjected transgene could be as high as 20%. Despite these initial successes, however, transgenic zebrafish had the problem of inconsistent expression of a transgene, i.e., variegated expression (Stuart *et al.*, 1990) or no expression (Stuart *et al.*, 1988; Culp *et al.*, 1991). In these experiments, promoter/enhancer sequences of SV40 or RSV (Rous sarcoma virus) origin were used. Lin *et al.* (1994b) used a *Xenopus* elongation factor 1α enhancer/promoter to drive LacZ expression. The expression patterns of LacZ in the four lines obtained were highly variable. Bayer and Campos-Ortega (1992) tried to apply an enhancer trap in zebrafish, using a mouse heat-shock promoter. Although one transgenic fish in which LacZ was expressed in primary sensory neurons was generated, LacZ expression was not fully penetrant. To date, the study by Amsterdam *et al.* (1995) appears to be the most successful in terms of the consistent expression of a reporter gene in zebrafish. They used a modified *Xenopus* elongation factor 1α enhancer/promoter to drive the expression of the green fluorescent protein (GFP) reporter gene (Chalfie *et al.*, 1994), and showed that five of five transgenic lines expressed GFP, apparently in whole bodies, although one line with the highest GFP expression still showed variegated GFP expression.

Recently, Hopkins and her colleagues developed a new method for generating transgenic zebrafish. They showed that murine leukemia virus/vesicular stomatitis virus pseudotyped retroviral vectors can integrate into the zebrafish genome at high frequency (Lin *et al.*, 1994a; Gaiano *et al.*, 1996a). The method has been proven to be useful for insertional mutagenesis (Gaiano *et al.*, 1996b; Allende *et al.*, 1996). However, since retroviral vectors have limitations of vector size, introduction into the genome of a relatively long construct, such as long *cis*-regulatory sequences, would be impossible using this method.

A possible cause for the frequently observed silent or variegated expression of the constructs introduced could, in part, be the use of sequences of heterologous origin. There is an example in which a promoter of zebrafish origin was used to drive the gene expression (Meng *et al.*, 1997). But it was a transient expression analysis; i.e., gene expression was monitored in an injected embryo, not in a germline-transmitted embryo (Meng *et al.*, 1997). To date, no transgenic zebrafish in which the gene is driven by a promoter of zebrafish origin, nor transgenic zebrafish in which a reporter gene is driven by a tissue specific promoter/enhancer, have been generated. Thus, whether the reproducible generation of transgenic zebrafish with tissue-specific transgene expression is possible has not yet been clarified. Given this background, we attempted to introduce GFP driven by a zebrafish muscle-specific actin promoter (*α-actin* promoter) into zebrafish to investigate whether reliable and tissue-specific expression in transgenic zebrafish can be achieved by using a tissue-specific promoter/enhancer of zebrafish origin. Here we show that it is possible to reliably obtain GFP-expressing transgenic zebrafish with the α-actin–GFP constructs. We also show that, by using zebrafish cytoskeletal *β-actin* promoter, it is possible to reliably obtain transgenic zebrafish which express GFP throughout the body.

In this study, we also investigated the effects of the following three factors which potentially affect transgenic frequency or expression levels. The first is whether levels of transient expression in an injected embryo show any correlation to transgenic frequency. The second is whether coinjection of the SV40 T antigen nuclear localization sequence, which has been suggested to facilitate the transfer of a transgene into embryonic nuclei (Collas *et al.*, 1996), has any effect on transgenic frequency. The third is whether the presence of a plasmid vector sequence has any effect on the expression levels of GFP.

MATERIALS AND METHODS

Cloning of Actin Genes from Zebrafish

RACE–PCR was carried out against first-strand cDNA of 14- to 24-h-old embryos using two degenerated primers based on the amino acid sequences, WHHTFY and WDDMEK, which are present in all types of actin proteins. The nucleotide sequences of the primers were TA(A/G)AA(A/G/C/T)CT(A/G)TG(A/G)TCCCA and GGAAGCTT(C/T)TCCAT(A/G)TC(A/G)TCCCA. Nested PCR was performed and four types of DNA fragments, about 300 bp each, were obtained.

An *α-actin* genomic fragment of about 3.7 kb, which includes the first and the second introns, was recovered by PCR using *α-actin*-specific primers. Southern blotting using this fragment as a probe revealed a single 12-kb band. A zebrafish genomic sublibrary was constructed using 10- to 15-kb *Eco*RI genomic fragments and screened with the 3.7-kb probe. Three identical positive clones, 12 kb in length, were isolated.

A *β-actin* genomic fragment of 1.9 kb, which includes the first and the second introns, was recovered by PCR using *β-actin*-specific primers. This fragment was used for the screening of zebrafish genomic libraries which were provided by Drs. Petkovich, Picker, Takeda, and Kikuchi. Several overlapping phage clones were obtained.

Construction of α-Actin–GFP and β-Actin–GFP Plasmids

Two modified GFP sequences, GFP-S65A (Moriyoshi *et al.*, 1996) and EGFP (Clontech), were used. An *α-actin* upstream fragment (*α-actin* promoter; *ap*) of about 3.9 kb was PCR-amplified from the *α-actin* λ phage clone using the upstream primer, T3, and the downstream primer, TTGGTCTGTGCAGGACAA. The junction between the first intron and the second exon exists within the downstream primer. The initiation methionine codon is located several nucleotides downstream of the downstream primer sequence. Two *ap*-GFP plasmids, *ap*-GFP(S65A) and *ap*-EGFP, were constructed. For the S65A construct, GFP(S65A) followed by an SV40 poly(A) signal was fused to *ap*. For the EGFP construct, EGFP followed by a BGH poly(A) signal was fused to *ap*. The backbone plasmid vec-

tors were pBluescript (SK) in both cases. Structural details of the two plasmids are available upon request.

A *β-actin* upstream fragment (*β-actin* promoter; *βp*) of about 17 kb was PCR-amplified from one of the *β-actin* phage clones using the upstream primer, T3, and the downstream primer, AAGGATCCACTGTAAAAGAAAGGGAA. The junction between the first intron and the second exon exists within the downstream primer. The initiation methionine codon is located several nucleotides downstream of the downstream primer sequence. For the poly(A) signal, a *β-actin* 3′ fragment (*β3′*) of about 8.4 kb was used. The upstream end of *β3′* is a *Bam*HI site, which is located within the second exon. The downstream end is an end of one of the phage clones. The exact location of the poly(A) additional signal is unknown. The *βp*-EGFP-*β3′* plasmid was constructed using the pBluescript (SK) backbone plasmid vector. Structural details of the plasmid is available upon request.

DNA Preparation and Microinjection

Plasmid DNA was prepared using the Qiagen plasmid kit (Qiagen). For BS-*αp*-G, the *αp*-GFP(S65A) plasmid was linearized by *Sal*I. For *αp*-G-BS, the *αp*-EGFP plasmid was linearized by *Sal*I. For *βp*-G-*β3′*-BS, the *βp*-EGFP-*β3′* plasmid was linearized by *Xho*I. Linearized plasmid DNA was extracted using phenol–chloroform and then chloroform, precipitated by ethanol, and dissolved in distilled water. For *αp*-G, the *αp*-GFP(S65A) plasmid was digested with *Sac*I and electrophoresed on an agarose gel. DNA fragments of *αp*-GFP(S65A)-pA were recovered from the gel using the GeneClean II kit (Bio 101) and dissolved in distilled water.

Maintenance of wild-type fish and collection of embryos were carried out as described by Westerfield (1993). Microinjection of DNA was carried out using an agarose gel with depressions as a holding plate (Westerfield, 1993). DNA solution of about 25 ng/μl in distilled water was air-pressure-injected into the cytoplasm of a one-cell-stage zebrafish embryo with its chorion intact. The SV40 T antigen nuclear localization sequence (NLS; CGGPKKKRKVG-NH_2) was added to the injection solution to a final concentration of 0.5 ng/μl in more than half of the experiments. The peptide was synthesized and HPLC-purified by Takara Co. In contrast to most previous studies, color dye, such as phenol red or fluorescein dextran, was not added to the injection solution. The injection volume was estimated from the difference in refractive indices between the injection solution and cytoplasm. The injection volume was adjusted such that one-third to half of the injected embryos died or became malformed by the next day; exact volume was unknown. Injections were usually carried out within 20 min after fertilization, when cytoplasm of embryos was not yet high. Fertilized eggs were collected several times (typically 3 times) for one injection session. We injected up to about 70 eggs per egg collection. Thus, when fish continued to produce eggs for 1 h, we were able to inject up to about 210 embryos. When fish produced all their eggs in a limited period, fewer embryos were injected. Usually, 100–150 embryos were injected in one injection session. The next day, live embryos exhibiting very weak GFP expression (such as fish-X in Fig. 3), about one-fourth, were discarded. About one-third of the fish selected for raising died before sexual maturity (usually within a few weeks). Usually 25–40 fish reached sexual maturity per typical injection session.

DNA Isolation and PCR

DNA isolation from pools of 50–200 3-day-old fish or from individual embryos was carried out according to Amsterdam *et al.* (1995). PCR reaction was carried out using two primers within GFP in either case of GFP(S65A) or EGFP, which resulted in the amplification of the 300-bp band from the respective plasmid. All reactions contained primers in the *islet2* gene (Tokumoto *et al.*, 1995) as an internal control. DNA isolation from adult fish for Southern blotting was carried out according to Westerfield (1993).

Fluorescence Microscopy

Embryos in a 9-cm plastic dish were observed using an Olympus IX70-FLA inverted fluorescence microscope with an FITC filter. Usually, the 4× objective lens was used. In some cases, photographs were obtained using an Olympus BX50-FLA noninverted fluorescence microscope.

RESULTS

Cloning of Actin Genes from Zebrafish

Four types of *actin* genes were identified by RACE–PCR (see Materials and Methods). They were called as *α1-*, *α2-*, *β-*, and *γ-actin* based on their expression patterns and the sequence similarity to *actin* genes from other species. *α1-actin* is also referred to simply as *α-actin*. The expression of *α(α1)*- and *α2-actin* was muscle-specific, while that of *β-* and *γ-actin* was ubiquitous. Figure 1 shows the expression patterns of *α-actin* (A and B) and *β-actin* (C and D).

Transient Expression of GFP in α-Actin–GFP-Injected Embryos

Genomic sequences flanking *α-actin* were isolated (see Materials and Methods). The structure of the 5′-region of *α-actin* is shown in Fig. 2A. The initiation methionine codon is located near the 5′ end of the second exon (20 bp downstream of the exon–intron junction). A DNA fragment of about 3.9 kb, which contains about 2.2 kb of the upstream fragment, about 40 bp of the first exon, about 1.7 kb of the first intron, and a part of the second exon (5 bp), was used as an *α-actin* promoter (*αp*). The first intron was included for two reasons: one is because intervening sequences have been suggested to increase gene expression in transgenic mice (Brinster *et al.*, 1988) and zebrafish (Amsterdam *et al.*, 1995) and the other is because there may be segments of *cis*-regulatory elements in the first intron. Modified GFP (S65A; Moriyoshi *et al.*, 1996), followed by the SV40 poly(A) signal, was fused to the *αp*. The *αp*-GFP(S65A) plasmid was linearized and injected into one-cell-stage embryos, which were later subjected to fluorescence analysis. Cytoplasmic injection of DNA constructs is known to result in mosaic expression of a reporter gene in zebrafish (e.g., Amsterdam *et al.*, 1995). As expected, fluorescence was observed in a subset of muscle cells in 1-day-old embryos (Fig. 3). Expression patterns were highly variable from embryo to embryo.

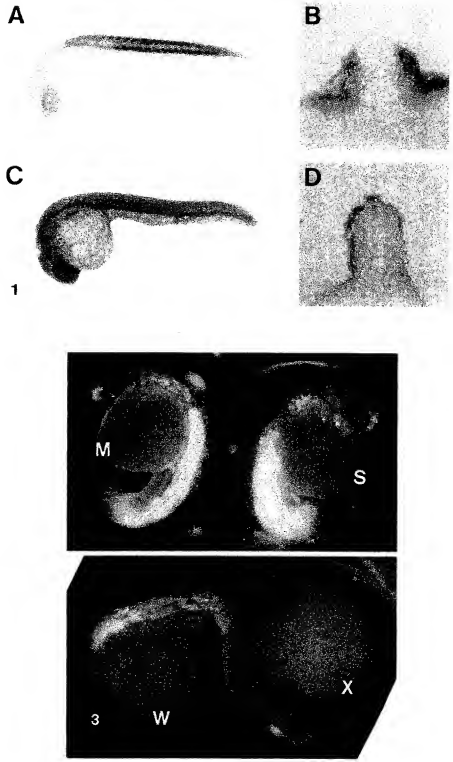


FIG. 1. Expression of *α-actin* and *β-actin* in embryonic zebrafish. One-day-old embryos were subjected to *in situ* hybridization using the *α-actin* (A and B) or *β-actin* (C and D) probe. A and C are whole mounts, while B and D are cross sections.

FIG. 3. Transient expression of GFP in *α*-actin–GFP-injected embryos. Embryos were viewed through their chorions. S, M, W, and X are the expression levels of GFP in each embryo. S, strong; M, moderate; W, weak; X, very weak, so that the embryo should be discarded.

FIG. 4. Expression of GFP in transgenic embryos generated using α-actin–GFP. Embryos were viewed through their chortons, except for (B) where chortons were removed. (A) 36-h-old embryo of a C-rank transgenic line. Note the absence of expression in the nontransgenic sibling. (B) 28-h-old embryos of C-rank and D-rank transgenic lines. A nontransgenic embryo is also shown. (C) 26-h-old embryo of a C-rank transgenic line. (D) A 13-h-old embryo of an A-rank transgenic line. (E) A 26-h-old embryo of the most fluorescent line (A-rank). Film-exposure time for this figure was much shorter than that in other figures. Due to the high fluorescence of the muscles, other regions of the embryo are clearly visible.

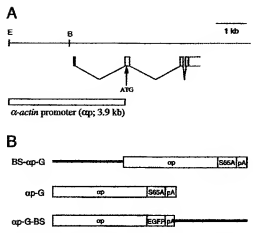


FIG. 2. Maps of *α-actin* and α-actin–GFP constructs used in this study. (A) Structure of *α-actin*. The initiation methionine codon (ATG) is located 20 bp downstream of the intron 1–exon 2 junction. The *α-actin* promoter used in this study (*αp*) includes the intron 1–exon 2 junction, but does not include the ATG initiation codon. E, *Eco*RI; B, *Bam*HI. (B) Structure of α-actin–GFP constructs used in this study. Thick line, plasmid vector sequence; S65A, GFP(S65A); pA, poly(A) additional signal.

Generation of Germline-Transmitting Fish Using α-Actin–GFP

Three constructs were used to produce stable transgenic zebrafish lines (Fig. 2B). BS-*αp*-G and *αp*-G were derived from the same plasmid, *αp*-GFP(S65A). The difference was the presence or the absence of plasmid vector sequence. The plasmid vector sequence has been suggested to suppress gene expression in transgenic mice (Chada *et al.*, 1985; Townes *et al.*, 1985). We also injected a third construct, *αp*-G-BS, in which EGFP (Clontech) was used instead of GFP(S65A) and the plasmid vector sequence was downstream of the fragment.

Each construct was injected individually into one-cell-stage embryos, which were raised for 1 day and viewed using an inverted fluorescence microscope. Embryos with few fluorescent cells were discarded. On average, about one-fourth of the injected embryos were discarded. In the case of BS-*αp*-G or *αp*-G, the remaining embryos were grouped according to the intensity of fluorescence. The purpose of this grouping is to investigate whether there is any correlation between levels of transient expression and the frequency of appearance of germline-transmitting founders. In more than half of the injections, the SV40 T antigen nuclear localization sequence (NLS; CGGPKKKRKVG-NH_2) was coinjected with DNA. This peptide has been suggested to facilitate the transfer of a transgene into embryonic nuclei (Collas *et al.*, 1996). To investigate the effects of NLS on the frequency of appearance of germline-transmitting founders, injections without NLS were also carried out.

Injected embryos were raised to sexual maturity and screened for germline-transmitting founders. The fish were mated to wild-type fish and the fluorescence of their 1-day-old progeny was examined using an inverted fluorescence microscopy. The frequency of appearance of germline-transmitting founders is summarized in Table 1, showing that germline-transmitting founders whose progeny expressed detectable levels of GFP were isolated in all types of experiments. In total, 41 of 194 (21%) were positive. As has been seen in previous studies of transgenic zebrafish lines (Stuart *et al.*, 1988, 1990; Culp *et al.*, 1991), founder fish had mosaic germlines. Rates of F1 inheritance of GFP-expressing offspring ranged from 2 to 50% (data not shown). For several lines ($n = 5$), DNA from individual offspring was extracted and analyzed for the presence of the GFP sequence by PCR. Every fluorescent embryo proved to be PCR-positive and every nonfluorescent embryo proved to be PCR-negative in all cases (data not shown). We also examined possible transgenic lines which did not express detectable levels of GFP. For this purpose, DNA was prepared from pools of embryos derived from each of those fish which only produced nonfluorescent embryos and analyzed by PCR. In to-

TABLE 1
Generation of Transgenic Zebrafish Using α-Actin–GFP

Construct	NLS	Exp	Frequency of germline transmitting founders with detectable levels of GFP expression		
BS-*αp*-G	+	S	1/3 (33%)	10/39 (26%)	
		M	6/24 (22%)		
		W	3/12 (25%)		
αp-G	+	S	4/26 (15%)	20/105 (19%)	41/194 (21%)
		M	9/41 (22%)		
		W	7/38 (18%)		
	−	S	3/10 (30%)	6/40 (15%)	
		M	1/9 (11%)		
		W	2/21 (10%)		
αp-G-BS	−	S/M	5/10 (50%)	5/10 (50%)	

Note. Exp, levels of transient GFP expression; S, strong; M, moderate; W, weak.

TABLE 2
Levels of GFP Expression in Transgenic Zebrafish Generated Using Each Construct

Fluorescence level / Construct	Bright A	B	C	Dim D
BS-αp-G	1	0	0	9
(total, 10)	(10%)	(0%)	(0%)	(90%)
αp-G	2	13	9	3
(total, 27*)	(7%)	(48%)	(33%)	(11%)
αp-G-BS	2	4	0	0
(total, 6*)	(33%)	(67%)	(0%)	(0%)

Note. One founder generated using αp-G and one founder generated using αp-G-BS produced progeny exhibiting two clearly different expression levels (A and C, A and B, respectively), which are individually classified and listed. Thus, the total numbers of αp-G and αp-G-BS (asterisks) are greater than those listed in Table 1.

tal, 123 fish were analyzed and 3 lines were PCR-positive. Considering that 41 of 194 fish were expression-positive founders, the results indicate that most, if not all, of the transgenic lines expressed detectable levels of GFP in the 1-day-old-embryo stage.

Figures 4A–4E show examples of GFP expression in transgenic lines. Except for one line expressing GFP throughout the body (data not shown), the lines (40 of 41) showed identical spatial expression patterns; GFP was expressed specifically in muscle cells. Variegated expression of GFP was not apparent in any of the lines. Thus, the results indicate that the zebrafish *α-actin* promoter can reliably drive the reporter gene expression in an identical manner as the endogeneous *α-actin* gene in transgenic zebrafish. This is the first demonstration of transgenic zebrafish in which the gene is driven by a tissue-specific promoter.

TABLE 3
Inheritance of α-Actin–GFP in Transgenic Zebrafish Lines

Construct	Line	Inheritance of GFP expression in F2
BS-αp-G	BAG-1	26/50 (52%)
αp-G	AG-1	188/374 (50%)
	AG-2	55/135 (41%)
	AG-3	125/240 (52%)
	AG-4	63/113 (56%)
	AG-5	40/72 (56%)
	AG-6	138/259 (53%)
	AG-7	165/306 (54%)
	AG-8	25/50 (50%)
	AG-9	34/68 (50%)
αp-G-BS	AGB-1	54/102 (53%)
	AGB-2	138/294 (47%)

In contrast to the identical spatial expression patterns of GFP, expression levels varied greatly from line to line. While some fluoresced only weakly (Fig. 4B), some fluoresced extremely strongly (Figs. 4C and 4E). The stage at which fluorescence was first detectable depended on the fluorescence intensity of each line. In the bright lines, GFP expression could be recognized at as early as 10 h, and at 13 h, clear fluorescence in the adaxial cells and somites was observed (Fig. 4D).

The fact that 21% (41 of 149) were GFP-expressing founders suggests that some founders might have multiple integrations. Indeed, at least two founders appeared to have two integrations, since they each produced progeny exhibiting two clearly different expression levels (see Note under Table 2). Southern analysis of F1 progeny from one such founder confirmed that two integrations had indeed occurred in the germline of the founder (data not shown).

Effects of Three Factors Which Potentially Affect Transgenic Frequency or Expression Levels

We examined the effects of three factors which may affect the transgenic frequency or expression levels. Results are summarized in Table 1 (transgenic frequency) and Table 2 (expression level). The first factor is the relationship between levels of transient expression and transgenic frequency. In two experiments using BS-αp-G (NLS+) and αp-G (NLS–), the transgenic frequency of the fish whose transient GFP expression had been strong (S) was higher than average, i.e., 33% (1/3) vs 26% (10/39) and 30% (3/10) vs 15% (6/40), respectively. However, in one experiment using αp-G (NLS+), it was lower than average, i.e., 15% (4/26) vs 19% (20/105). Importantly, transgenic frequency in W (weak) class fish was not very low compared with the average, i.e., 25% (3/12) vs 26% (10/39), 18% (7/38) vs 19% (20/

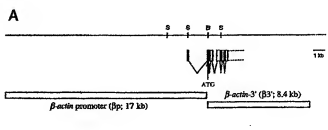


FIG. 5. Maps of *β-actin* and a β-actin–GFP construct used in this study. (A) Structure of *β-actin*. The initiation methionine codon (ATG) is located 8 bp downstream of the intron 1–exon 2 junction. The *β-actin* promoter used in this study (βp) includes the intron 1–exon 2 junction, but does not include the ATG initiation codon. S, *Sal*I; B, *Bam*HI. (B) Structure of a β-actin–GFP construct used in this study. Thick line, plasmid vector sequence.

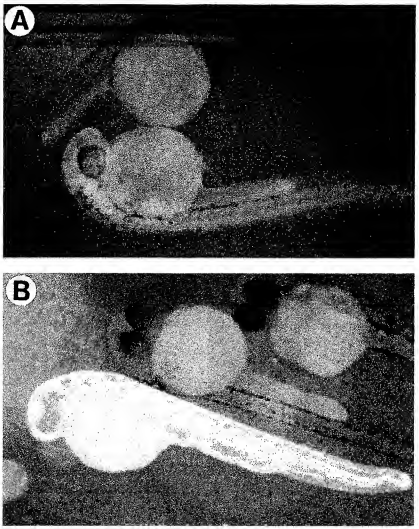


FIG. 6. Expression of GFP in transgenic embryos generated using β-actin–GFP. Chorions were removed. Nonfluorescent embryos are nontransgenic siblings. (A) A typical transgenic embryo at 28 h. (B) A transgenic embryo from the most fluorescent line at 28 h.

105), and 10% (2/21) vs 15% (6/40) in the three experiments. Thus, if there is any correlation, it is not so high that the W class fish should be discarded.

The second one is whether the NLS peptide contributes to an increase in transgenic frequency. NLS+ and NLS− experiments were conducted using the same construct, αp-G. The transgenic frequency in the NLS+ experiment was 19% (20/105), which was somewhat higher than that in the NLS− one, 15% (6/40). However, the difference was not so great that the result was concluded to be positive. Further study is required to determine whether the NLS peptide contributes to transgenic frequency.

The third one is whether the plasmid vector sequence affects the reporter gene expression. Each line was classified into ranks from A–D according to the fluorescence intensity of F1 progeny (Table 2). For the BS-αp-G construct, 9 of 10 lines were classified into the dim D rank, while in the case of the αp-G construct, the D rank was rather rare (3 of 27) and most of the lines were classified into B or C ranks (22 of 27). Considering that BS-αp-G and αp-G were derived from the same plasmid, the results suggest that the plasmid vector sequence has an adverse effect on expression. For the αp-G-BS construct, however, all of the lines (6 of 6) were classified into A or B ranks, despite the presence of the plasmid vector sequence. The high fluorescence was not due to the difference in the GFP sequences used (S65A and EGFP), since mRNA expression levels were also high in the 6 lines generated with the αp-G-BS construct. Thus, the result suggests that the plasmid vector sequence located downstream of the construct does not have an adverse effect on expression.

Maintenance of GFP Expression over a Generation

It is important to determine whether GFP expression is stable after passage through a germline. Thus, fluorescent progeny (F1) of each founder were raised to sexual maturity and mated with wild-type fish. All the lines tested (n = 24) produced fluorescent embryos. Moreover, levels of GFP expression were also completely inherited, i.e., the fluorescence intensity of the embryos (F2 progeny) was the same as that of the parents (F1) when they had been tested for fluorescence. As in previous studies, inheritance of fluorescence in the F2 generation was consistent with the ratio of Mendelian segregation in all the 12 lines where the number of fluorescent and nonfluorescent embryos were counted (Table 3). In three lines, stable transmission of GFP expression has been confirmed in the F3 generation. Taken together, the results strongly support the idea that the transgene is stably integrated into the genome in each line.

F1 progeny of B–D ranks were all healthy. In the case of A rank, a significant fraction of embryos died within 2 weeks. For the reason of the weakness, long exposure of excitation light to A-ranked embryos on a fluorescence microscope could be harmful to embryonic health. However, this is not the only cause of the weakness because those embryos which had been placed in a dark without excitation were still weak. Whether high-level expression GFP itself has toxic effects remains to be determined. If fish could survive in a critical period (a few weeks), most of them were able to become adults and to produce progeny. However, we have not yet succeeded in raising F1 progeny of two lines with extreme GFP expression (Fig. 4E).

Generation of Germline-Transmitting Fish Using β-Actin–GFP

We also generated transgenic zebrafish using the β-actin–GFP construct. A physical map of the zebrafish *β-actin* gene and the construct of the β-actin–GFP transgenic vector are shown in Fig. 5 (for details, see Materials and Methods). We obtained 4 founders out of 53 fish (8%). Three of the four lines exhibited similar GFP expression patterns. GFP was expressed throughout the body in a manner identical to that of the *β-actin* gene (Fig. 6A). In the remaining one line, GFP was also expressed throughout the body, and fluorescence was more intense than that of the other lines (Fig. 6B). In addition, a more prominent expression in the notochord was observed (Fig. 6B). In all of the lines, variegated expression was not observed. Transmission of GFP expression has been confirmed in the F2 generation in two lines.

In summary, we conclude that the *β-actin* genomic sequences used here lead to the reporter gene expression in an identical or near-identical manner to that of the endogeneous *β-actin* gene.

DISCUSSION

Consistent GFP-Expression of Transgenic Zebrafish Generated Using α-actin–GFP or β-actin–GFP Constructs

A number of transgenic zebrafish have been generated in the past 9 years. In the early phase, transgenic zebrafish suffered from either silencing of the transgene expression (Stuart *et al.*, 1988; Culp *et al.*, 1991) or nonconsistent (variegated or highly variable) expression (Stuart *et al.*, 1990; Lin *et al.*, 1994b). More consistent expression was observed by Amsterdam *et al.* (1995), who found that five of five transgenic lines showed near ubiquitous GFP expression in one construct (modified *Xenopus* ef1α enhancer/promoter and the rabbit *β-globin* second intron). In all of the previous studies, promoter/enhancer sequences were not derived from zebrafish origin. There have been no reports of transgenic zebrafish generated using a gene driven by a tissue-specific promoter/enhancer. With this background, we isolated a zebrafish muscle-specific actin promoter and generated transgenic zebrafish using α-actin–GFP constructs. The main aim of this study was to determine whether transgenic zebrafish exhibiting tissue-specific expression can be consistently generated with the use of zebrafish-origin promoters. We have generated as many as 41 GFP-expressing transgenic lines using α-actin–GFP constructs. In virtually all lines established, GFP was expressed in a nearly identical manner to the endogeneous genes; i.e., GFP was specifically expressed in muscle cells. Non-GFP-expressing transgenic lines were extremely rare. These results demonstrate that transgenic zebrafish with consistent expression can be reliably generated using the zebrafish *α-actin* promoter. We also isolated and used the zebrafish *β-actin* promoter. Transgenic zebrafish exhibiting GFP expression throughout the body were reliably generated in this case. This further supports the hypothesis that consistent expression can be achieved by the use of zebrafish-origin promoters.

Establishment of transgenic zebrafish lines using the α-actin–GFP construct is quite easy with the aid of reliable detection of the transgene expression *in vivo*. Screening of one line by merely observing about a hundred embryos through their chorions on an inverted fluorescence microscope usually takes only a few minutes. Transgenic fish can be easily detected among many nontransgenic siblings. Preliminary experiments showed that, at least in transient expression assays, the α-actin–GFP sequence did not affect the expression of another transgene which was driven by several other promoters, when they were placed in the same plasmid. Thus, the addition of α-actin–GFP to one's own constructs could provide a useful *in vivo* marker in the generation of transgenic zebrafish.

In conclusion, we have shown that it is, in principal, possible to consistently generate transgenic zebrafish exhibiting tissue-specific expression of a reporter gene. Since zebrafish has become a popular model organism for the study of vertebrate development, the generation of transgenic zebrafish which express a reporter gene in specific tissues or cells will undoubtedly be useful in the future. In particular, the transparency of the zebrafish embryo will make GFP an excellent marker.

Factors Which Potentially Affect Transgenic Frequency or Expression Levels

We have determined the effects of three factors (levels of transient expression, presence of the NLS peptide, and

presence or location of the plasmid vector sequence) which potentially affect the transgenic frequency or expression levels of the transgene. Levels of transient expression the day after injection did not show strong correlation with transgenic frequency if one-fourth of the embryos, those exhibiting too weak an expression level, were eliminated. The effect of the NLS peptide on transgenic frequency remained to be elusive. The location of the plasmid vector sequence greatly influenced the expression levels of the transgene. Our study suggests that a plasmid vector sequence placed upstream of a transgene has negative effects. In summary, our proposed procedure for dealing with these factors is as follows. First, on monitoring the transient expression in transgenic zebrafish, only embryos exhibiting very weak expression should be discarded. The NLS peptide may be coinjected. If the plasmid vector sequence is not removed, it should be placed at the 3′ end of the construct. We have been able to reproducibly obtain 20–30% GFP-expressing transgenic founders ($n = 29$ for founders) using other α-actin–GFP-based constructs, under these conditions (S.H., unpublished observation).

We should point out the possibility that the plasmid vector sequence may not be the main cause of the difference in the expression levels. Some other factors, such as the kind of restriction enzymes used or a specific DNA sequence near either end of the construct, may affect the copy number or integration site and thus eventually affect expression levels. Thus, although our results suggest that the plasmid vector sequence should be removed or placed downstream of a transgene, more work is required to further clarify the effects of the plasmid vector sequence.

The transgenic frequency of αp-G was significantly lower than the other two, BS-αp-G and αp-G-BS. This may suggest that the removal of the plasmid vector sequence decreases the transgenic frequency. Decrease of the DNA quality, which could be induced by the procedure of the removal of the plasmid vector sequence, may be responsible for this.

In this study, the frequency of appearance of germline-transmitting founders using the α-actin–GFP constructs was considerably high (in total, 21%) compared to that in previous studies (on average, 5–10%). One possible reason for this could be that embryos exhibiting very weak GFP expression in the transient expression assay were discarded. Through that procedure, we were probably able to eliminate those embryos with insufficient amount of injected DNA. Minor modifications in the injection procedure (see Materials and Methods) could, in part, also be responsible. The length of injected DNA may considerably affect the transgenic frequency, since the transgenic frequency for the β-actin–GFP, which is four times longer than α-actin–GFP, was 8%. The DNA sequence itself could also affect the transgenic frequency, since Culp *et al.* (1991) reported that the frequency could be as high as 20% for one construct. To determine which are the main contributors to the high-frequency generation of transgenic zebrafish using the α-actin–GFP constructs, further study is required.

While this paper was in review, we noticed that the group of Lin also succeeded in generating transgenic zebrafish exhibiting tissue-specific GFP expression using the zebrafish *GATA-1* promoter (Long *et al.*, 1997).

ACKNOWLEDGMENTS

We thank M. Sugiura for assistance with the experiments. We also thank K. Moriyoshi and S. Nakanishi for providing GFP-S65A, M. Petkovich, A. Picker, H. Takeda, and Y. Kikuchi for providing zebrafish genomic libraries, and K. Takarnatsu and Y. Katoh for maintaining our fish colony. This work was supported by a grant from JST to S.H.

REFERENCES

Allende, M. L., Amsterdam, A., Becker, T., Kawakami, K., Gaiano, N., and Hopkins, N. (1996). Insertional mutagenesis in zebrafish identifies two novel genes, *pescadillo* and *dead eye*, essential for embryonic development. *Genes Dev.* **10**, 3141–3155.

Amsterdam, A., Lin, S., and Hopkins, N. (1995). The *Aequorea victoria* green fluorescent protein can be used as a reporter in live zebrafish embryos. *Dev. Biol.* **171**, 123–129.

Bayer, T. A., and Campos-Ortega, J. A. (1992). A transgene containing *lacZ* is expressed in primary sensory neurons in zebrafish. *Development* **115**, 421–426.

Brinster, R. L., Allen, J. M., Behringer, R. R., Gelinas, R. E., and Palmiter, R. D. (1988). Introns increase transcriptional efficiency in transgenic mice. *Proc. Natl. Acad. Sci. USA* **85**, 836–840.

Chada, K., Margram, J., Raphael, K., Radice, G., Lacy, E., and Costantini, F. (1985). Specific expression of a foreign beta-globin gene in erythroid cells of transgenic mice. *Nature* **314**, 377–380.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., and Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* **263**, 802–805.

Collas, P., Husebye, H., and Alestrom, P. (1996). The nuclear localization sequence of the SV40 T antigen promotes transgene uptake and expression in zebrafish embryo nuclei. *Transgen. Res.* **5**, 451–458.

Culp, P., Nüsslein-Volhard, C., and Hopkins, N. (1991). High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. *Proc. Natl. Acad. Sci. USA* **88**, 7953–7957.

Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J., and Boggs, C. (1996). A genetic screen for mutations affecting embryogenesis in zebrafish. *Development* **123**, 37–46.

Gaiano, N., Allende, M., Amsterdam, A., Kawakami, K., and Hopkins, N. (1996a). Highly efficient germ-line transmission of proviral insertions in zebrafish. *Proc. Natl. Acad. Sci. USA* **93**, 7777–7782.

Gaiano, N., Amsterdam, A., Kawakami, K., Allende, M., Becker, T., and Hopkins, N. (1996b). Insertional mutagenesis and rapid cloning of essential genes in zebrafish. *Nature* **383**, 829–832.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J. M., Jiang, Y.-J., Heisenberg, C.-P., Kelsh, R. N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., and Nüsslein-Volhard. (1996). The identification of genes with unique and es-

sential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1–36.

Kimmel, C. B. (1989). Genetics and early development of zebrafish. *Trends Genet.* **5**, 283–288.

Lin, S., Gaiano, N., Culp, P., Burns, J. C., Friedmann, T., Yee, J-K., and Hopkins, N. (1994a). Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. *Science* **265**, 666–669.

Lin, S., Yang, S., and Hopkins, N. (1994b). *lacZ* expression in germline transgenic zebrafish can be detected in living embryos. *Dev. Biol.* **161**, 77–83.

Long, O., Meng, A., Wang, H., Jessen, J. R., Farrell, M. J., and Lin, S. (1997). *GATA-1* expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. *Development* **124**, 4105–4111.

Meng, A., Tang, H., Ong, B. A., Farrell, M. J., and Lin, S. (1997). Promoter analysis in living zebrafish embryos identifies a cis-acting motif required for neuronal expression of GATA-2. *Proc. Natl. Acad. Sci. USA* **94**, 6267–6272.

Moriyoshi, K., Richards, L. J., Akazawa, C., O'Leary, D. D. M., and Nakanishi, S. (1996). Labeling neural cells using adenviral gene transfer of membrane-targeted GFP. *Neuron* **16**, 255–260.

Nusslein-Volhard, C. (1994). Of flies and fishes. *Science* **266**, 572–574.

Stuart, G. W., McMurray, J. V., and Westerfield, M. (1988). Replication, integration and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. *Development* **103**, 403–412.

Stuart, G. W., Vielkind, J. R., McMurray, J. V., and Westerfield, M. (1990). Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. *Development* **109**, 577–584.

Tokumoto, M., Gong, Z., Tsubokawa, T., Hew, C. L., Uyemura, K., Hotta, Y., and Okamoto, H. (1995). Molecular heterogeneity among primary motoneurons and within myotomes revealed by the differential mRNA expression of novel *Islet-1* homologs in embryonic zebrafish. *Dev. Biol.* **171**, 578–589.

Townes, T. M., Lingrel, J. B., Brinster, R. L., and Palmiter, R. D. (1985). Erythroid specific expression of human beta-globin genes in transgenic mice. *EMBO J.* **4**, 1715–1723.

Westerfield, M. (1993). "The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Brachydanio rerio*)." Univ. of Oregon Press, Eugene, OR.

Received for publication August 19, 1997
Accepted October 13, 1997

EXHIBIT 7

Vol. 211, No. 2, 1995
June 15, 1995

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 438-446

C45

DETERMINATION OF A NECDIN CIS-ACTING ELEMENT REQUIRED FOR NEURON SPECIFIC EXPRESSION BY USING ZEBRA FISH

C.-H. Kuo#, T. Uetsuki*, C.-H. Kim, H. Tanaka, B.-S. Li, E. Taira, H. Higuchi, H. Okamoto, K. Yoshikawa* and N. Miki**

Department of Pharmacology I, Medical School, * Institute for Protein Research, Osaka University. 2-2 Yamadaoka, Suita, Osaka 565, Japan

** Department of Physiology, Keio University School of Medicine, Tokyo, Japan

Received May 7, 1995

To determine cis-acting elements required for neuron specific expression of a necdin gene, we tried to use zebra fish assay system *in vivo* instead of cell lines in vitro. Various expression vectors carrying upstream sequences of necdin gene fused to MEKA (lacZ) gene as a reporter were injected into fertilized zebra fish embryos and then the expression of the reporter gene was analyzed by the whole mount immunochemical method. No promoter activity was obtained with a construct carrying sequence from -63 to +63 of the necdin gene, while promoter activity with preferential skin expression was obtained with a construct having sequence from -86 to +28. Further upstream sequence from -173 to +28 exhibited neuron specific expression as well as that from -845 to +63. These results indicate that a cis-acting element responsible for neuron specific expression is located in an 87bp sequence from -173 to -87 of necdin gene.

A novel necdin cDNA is isolated from P19 embryonal carcinoma cells by the differential hybridization method between untreated and retinoic acid treated cells which are differentiated to neural cells. It encodes 325 amino acids whose molecular size and pI value are about 37 kDa and 8.3, respectively (1, 2). The necdin cDNA as a probe only hybridizes with 1.7kb mRNA of the brain among mouse tissues tested and the differentiated P19 cells, indicating neuron specific expression of necdin (1, 2). *In situ* hybridization experiment also supports the

To whom correspondence should be addressed. Fax : 06-879-3529.

0006-291X/95 $5.00

neuron specific expression (3). Immunohistochemical analysis reveals that necdin protein is a neuron specific nuclear protein whose expression is seen in postmitotic cells (1, 2). Although the physiological function of necdin protein is unknown, it is interesting to investigate cis-acting elements required for neuron specific expression of necdin, because it is expected to elucidate the mechanism underlying neurogenesis and neuronal differentiation through the transcriptional regulation. Uetsuki et al. (3) have cloned genomic necdin DNA which shows an intronless structure. Transfection of a plasmid carrying an upstream sequence of the necdin gene (from -845 to +63) fused to lacZ gene induce the expression of β-galactosidase in retinoic acid treated P19 cells (3), suggesting that the above upstream sequence contains a region required for neuron specific expression.

Recently, zebrafish, *Brachydanio rerio*, is emerging as a model organism as well as Drosophila and Xenopus laevis. A genetic linkage map for zebrafish and several neuronal mutants have been reported (4, 5). Zebrafish is a useful animal for neuroscience as following reasons ; 1) breeding is easy. 2) fertilized embryos are available every day. 3) primary neurons are formed within 24hr. 4) whole mount analysis is easy, because of the lucid body. 5) it is possible to inject plasmid DNA into more than one hundred of fertilized embryos. By using zebrafish, analysis of GAP-43 promoter activity have been challenged (6). We also attempted to use zebrafish for analyzing cis-acting DNA elements required for neuron specific expression of necdin gene.

MATERIALS AND METHODS

The zebra fish: The Oregon AB line, were maintained at 28.5℃ as described in (7). Expression vector suspended in 25 μg/ml of 2mM Na-phosphate buffer, pH 7.4, 10 mM KCl, 0.01% phenol red and 0.2% ethanol was injected into about 150 fertilized embryos about 500pl in one experiment by Narishige microinjector. Analysis of transgene expression was carried out as described by Reinhard et al (6). Efficiency to produce transient transgenic fish and positive cells numbers per embryo with various constructs were 5.8-18.3% and 1-32 cells, respectively, which

are in a range of reported experiment (6). In this experiment, the above values and transcription level were decreased with progressive deletion of 5' upstream region of the necdin gene.

Preparation of various necdin promoter/enhancer gene plasmids: Necdin promoter, both orientations (Construct 9 and 10 in Fig. 1), extended from -845 to + 63 fused to lacZ gene were constructed at HindIII site in pBluescript SK(-) plasmid (3). Additional constructs containing MEKA cDNA (8) as a reporter gene was prepared as follows; Expression plasmid pcDNAI having CMV promoter (Pcmv) was digested with EcoRI/EcoRV at polylinker site. It was ligated with MEKA cDNA encoding a full length of MEKA protein, and used as a control (Pcmv-MEKA/pcDNAI). The control vector was digested with AflIII, blunt ended and ligated with HindIII linker. It was digested again with HindIII in the presence or absence of BamHI to remove Pcmv and then ligated with HindIII digested necdin promoter (from -845 to +63) prepared from the pBluescript SK(-) or with HindIII/BamHI digested PCR products carrying various length of the necdin promoters. Synthetic oligonucleotide primers having HindIII site or BamHI site for PCR were as follows ; 5' GCAAGCT (-291 of necdin gene)TGCCC AAACTCAGTGTGTCCG, 5' GCAAGC (-173)TTTACATAGCCTACTGGTACC, 5' GCAAG (-123) CTTTGACTCTTCGGCTCCTTTC, 5'GCAAGC(-86)TT CTGG CTTCCCCAACACGCA, 5'GCGGAT(+28)CCCTCGGTGGAGACCAGCAG, and 5' GC GGATCCT (+1) GCGCTTTACTGAGCACTGCG.

Whole mount immunochemical procedures : It was performed according to the methods reported (9,10) with a slightly modification. Briefly, to remove the chorion, embryo was treated with 1mg/ml of pronase for 2min at room temperature and fixed with 4% paraformaldehyde containing 0.2% glutaraldehyde, 4% sucrose, 0.15mM $CaCl_2$ and 0.1M Na-phosphate buffer (pH 7.4) for more than 4hr. It was washed with 100 mM Na-phosphate buffer (pH 7.4) containing 0.85% NaCl and 0.3% triton X-100, and then with PBST (20 mM Na-phosphate buffer, pH 7.4, 0.85% NaCl and 0.3% triton X-100) for 60min. To reduce back ground, the sample was further incubated with PBSTS (PBST containing 0.5% skim milk) for 60min. After over night incubation at 4℃ with anti-MEKA, anti-β-galactosidase (both antisera did not react with endogeneous proteins in normal zebrafish embryo) or anti-HNK serum in PBSTS, the sample was washed with PBSTS. Second antibody conjugated with horseradish peroxidase was reacted and then washed with PBSTS, PBST and PBS/0.1% triton X-100. Antigen in embryo was visualized indirectly with 0.1mg/ml of DAB in PBS/0.1% triton X-100 in the presence of 0.05% hydrogen peroxide. The sample was treated with methanol and then benzyl benzoate : benzyl alcohol (2:1), and provided for photograph.

RESULTS AND DISCUSSIONS

Transfection of a plasmid containing specific promoter region into various cell lines and subsequent determination of a reporter gene expression is the well accepted method for the determination of promoter/enhancer region. However, it has been reported that when neurofilament (NF) promoter fused to lacZ gene is transfected into NF-producing PC12h cells or NF-nonproducing C6 cells, C6 cells

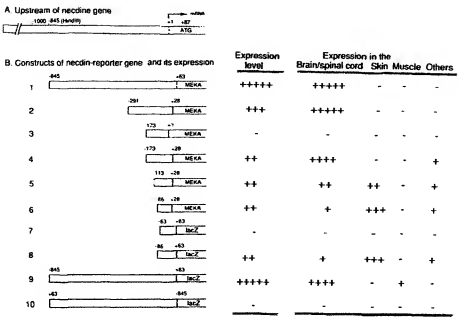


Fig. 1. Summary of the expression level and tissue specific expression directed by various constructs of necdin promoter/reporter gene from 3-6 experiments. Upstream of necdin gene with HindIII site and ATG codon (A) and constructs of necdin-reporter gene and its expression in embryos at 24hr (B) are illustrated. Construct 1 was used as a control of expression level (+++++ : as a 100%). The tissue specific expression found in the brain/spinal cord, skin, muscle and others was calculated from total stained cell numbers and illustrated as + (0-20%) to +++++ (80-100%).

express 40 times higher β-galactosidase activity than the PC12h by unknown mechanism (11). In transgenic mice, however, the NF promoter directs neuron specific expression of CAT-reporter gene (12). The results indicate that analysis of promoter region *in vitro* by using cell lines does not always reveal tissue specific gene expression. Therefore, we attempted to utilize zebrafish for determining neuron specific element of necdin gene as an *in vivo* assay system.

At first, various constructs (Construct 1 to 10 in Fig. 1B), carrying different length of the necdin promoter region fused to MEKA or lacZ gene as a reporter, were prepared to identify cis-acting elements responsible for neuron specific expression. The cis-acting elements are expected to exist in the 5' flanking region,

because of the intronless structure of the necdin gene (3). The constructs were injected into fertilized zebrafish embryos and tissue specificity was assayed by counting the cell numbers expressing the reporter protein in different tissues. The results including expression level were summarized in Fig. 1B.

We injected Constructs 1 (MEKA as a reporter) and Construct 9 (lacZ), both of which contain from -845 to +63 of the necdin gene in each expression vector, into fertilized zebrafish embryos and assayed the reporter gene expression in 24hr embryos by the whole mount immunochemical method. Although mosaic expression pattern was observed, more than 80% among the stained cells were restricted to the primary sensory neurons, Rohon-Beard cells and trigeminal ganglion neurons, and neurons in the brain (data not shown). They are dominant neurons which are formed in early developmental stage and their location were confirmed by staining them with anti-HNK serum as a neural cell membrane marker (13). On the other hand, the expression directed by Pcmv-MEKA/pcDNAI as a control vector was observed preferentially in the skin and less than 10% in the primary sensory neurons (data not shown). During the investigation, we noticed that the injection of more than 100μg/ml concentration of vectors resulted in malformation significantly, and also the linearized or freeze-thawed vectors resulted in the reduction of neuron specific expression with increasing of skin expression (data not shown).

Next, we examined the time-course of the reporter gene expression with Construct 1. Embryos were fixed at 22, 24 and 28hr and then analyzed. Additional neural cells, motoneurons and interneurons were stained in 28hr embryos (Fig. 2), compared to those in 22 or 24 hr embryos which were mentioned above. These results indicate that mouse necdin promoter region spanning from -845 to +63 functioned in zebrafish embryos in temporal, spatial and tissue specific manner. The

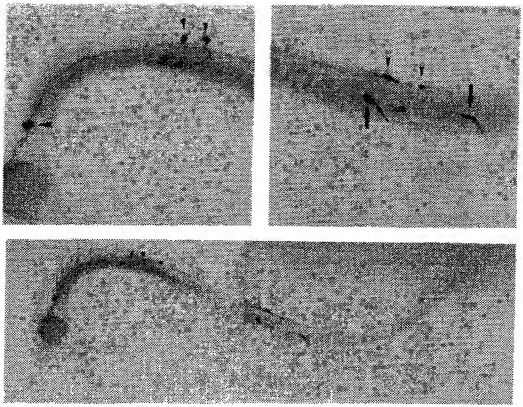


Fig. 2. Expression of MEKA protein directed by necdin promoter in zebra fish. Necdin promoter extended from -845 to +63 fused to MEKA cDNA as a reporter gene was prepared (Construct 1) and MEKA expression was assayed in zebra fish at 28hr. One of the typical embryos reveals 23 positive cells consisted of 22 neurons with neurites and one unidentified cell. Trigeminal ganglion neuron, Rohon-Beard cell, motoneuron and interneuron are indicated by arrowhead, small arrowhead, arrow and small arrow, respectively, in upper enlarged photographs.

promoter activity was dependent on direction of the cis-element, since Construct 10 with a inverted sequence showed no promoter activity.

In the present study, MEKA cDNA is used as a reporter gene. The MEKA protein, identical to phosducin (14), form a complex with $\beta\gamma$ subunits (15,16) of guanine nucleatid-binding proteins, Gt , Gs or Gi. Therefore, the expression of MEKA protein is speculated to inhibit the neurite outgrowth through increasing free $Go\alpha$ concentration in growth cone (17). However, we could not detect any significant morphological changes in zebrafish neural cells and neurites that

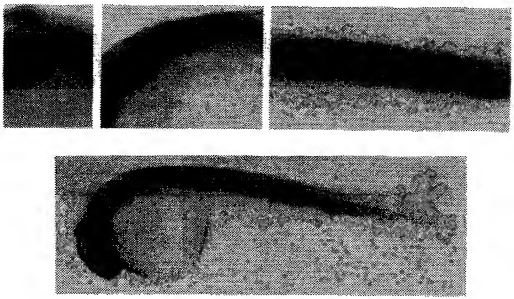


Fig. 3. Neuron specific expression induced by necdin promoter extended from -173 to +28. Expression vector containing necdin promoter (-173 to +28) fused to MEKA cDNA (Construct 4) was injected into fertilized zebra fish embryos and assayed at 24hr. One of the typical embryos reveals 13 positive neurons with neurites.

expressed MEKA protein. One of the possible explanations may be the inability of MEKA protein to associate with $\beta\gamma$ subunits of Go.

Expression level of the reporter gene, which reflects the transcriptional activity, was gradually decreased with progressive deletion of 5' flanking region of the necdin promoter (Constructs 1 to 6 in Fig. 1 corresponding to -845 to - 86), and no exppression was observed with Construct 7 (from -63 to +63) in accordance with those of the Uetsuki et al (3). A 5' flanking region from +63 to at least +28, but not to +1, was necessary to retain the promoter activity (compare with Constructs 3 and 4) and may be involved in ribosomal RNA binding.

The shortest fragment required for neuron specific expression among the tested plasmids was observed in Construct 4 (from -173 to +28) as shown in Fig. 1 and

3. In conjunction with the result that Construct 6 (from -86 to +63) showed the promoter activity with preferential skin expression, we concluded that the cis-acting element should locate in an 87bp sequence from -173 to -87 of necdin gene. No homologous consensus sequence was found in the 87bp from known DNA elements including the neuron-restrictive silencer element (NRSE) reported by Mori et al (18). Physiological function of six CTTX repeats found in the 87bp of necdin gene is unknown.

It is interesting to note that Constructs 6 and 8 (from -86 to +63 and from -81 to +63, respectively) almost lost the neuron specific expression in spite of skin expression in this study. although the Construct 8 still causes neuron specific expression in the *in vitro* assay system using retinoic acid treated P19 cells (3). It is suspected that the association of several proteins interacting with multiple DNA elements within the 87bp of the necdin gene is necessary for producing neuron specific expression, because further 50bp deletion (Construct 5 in Fig. 1) from the 5' region of the necdin gene (Construct 4) still exhibited preferential neural expression. Therefore, the different results between *in vitro* and *in vivo* assay systems may be due to the lack of protein(s) which interacts with multiple DNA sites. Difference between Constructs 4 and Constructs 5/6 seems to involve in a switch from neuron specific to preferential skin expression (Fig. 1), indicating the possibility that there is another regulatory element(s) from -173 to -87 for regulating neuron specific expression.

In the present report. we demonstrated by the analysis of necdin gene that zebrafish is a useful screening system to determine a unique enhancer/promoter region, especially an element required for neuron specific expression, because of a rapid development of the primary neurons in a day.

REFERENCES

1) Maruyama, K., Usami, M., Aizawa, T., and Yoshikawa, K. (1991) Biochem. Biophys. Res. Commun., 178, 291-296
2) Aizawa, T., Maruyama, K., Kondo, H., and Yoshikawa, K. (1992) Dev. Brain Res., 68, 265-274
3) Uetsuki, T., and Yoshikawa, K. In preparation
4) Postlethwait, J. H., Johoson,S.L., Midson,C.N., Talbot,W.S., Gates,M., Ballinger, E. W., Africa, D., Andrews, R., Carl, T., Eisen, J. S., Horne, S., Kimmel, C. B., Hutchinson, M., Johnson, M., and Rodriguez, A. (1994) Science, 264, 699-703
5) Kahn, P. (1994) Science, 264, 904-905
6) Reinhard, E., Nedivi, E., Wegner, J., Skene, J. H. P., and Westerfield, M. (1994) Development, 120, 1767-1775
7) Westerfield., M. (1992) The Zebrafish Book, 2nd edition. University of Oregon Press
8) Kuo, C.-H., Akiyama, M., and Miki, N. (1989) Mol. Brain Res., 6, 1-10
9) Westerfield., M., Wegner, J., Jegalian, B. G., DeRobertis, E. M., and Puschel, A. W. (1992) Genes & Dev. 6, 591-598
10) Brivanlou, A. H., and Harland, R. M. (1989) Development, 106, 611-617
11) Nakahira, K., Ikenaka, K., Wada, K., Tamura, T., Furuichi, T., and Mikoshiba, K. (1990) J. Biol. Chem., 265, 19786-19791
12) Reeben, M., Halmekyto, M., Alhonen, L., Sinervirta, R., Saarma, M., and Janne, J. (1993) Biochem. Biophys. Res. Commun., 192, 465-470
13) Metcalfe, W. K., Myers, P. Z., Trevarrow, B., Bass, M. B., and Kimmel, C. B. (1990) Development, 110, 491-504
14) Lee, R. H., Fowler, A., McGinnis, J. F., Lolley, R. N., and Craft, C. M. (1990) J. Biol. Chem., 265, 15867-15873
15) Kuo, C.-H., Taniura, H., Watanabe, Y., Fukada, Y., Yoshizawa, T., and Miki, N. (1989) Biochem. Biophys. Res. Commun., 162, 1063-1068
16) Jaffe, L. A., Gallo, C. J., Lee, R. H., Ho, Y-K., and Jones, T. L. (1993) J. Cell Biol., 121, 775-783
17) Strittmatter, S. M., Valenzuela, D., Kennedy, T. E., Neer, E. J., and Fishman, M. C. (1990) Nature, 344, 836-841
18) Mori, N., Schoenherr, C., Vandenbergh, D. J., and Anderson, D. J., (1992) Neuron, 9, 45-54

EXHIBIT 8

Neurochemical Research, Vol. 21, No. 2, 1996, pp. 231–237

C44

Neuron-Specific Expression of a Chicken Gicerin cDNA in Transient Transgenic Zebrafish*

Cheol-Hee Kim,[1] Eiichi Taira,[1] Che-Hui Kuo,[1] Bing-Sheng Li,[1] Hitoshi Okamoto,[2] Kensuke Nakahira,[3] Kazuhiro Ikenaka,[3] Hiroshi Higuchi,[1] and Naomasa Miki[1,4]

(Accepted October 25, 1995)

Gicerin, a novel cell adhesion molecule which belongs to the immunoglobulin superfamily, is expressed temporally and spatially in the developing chick brain and retina. The previous in vitro experiments using transfected cells showed that gicerin can function as a cell adhesion molecule which has both homophilic and heterophilic binding activities. For the in vivo analyses of gicerin in neural development, we tried to utilize a zebrafish system, a vertebrate suitable for studying early development. We generated transient transgenic animals by microinjecting DNA constructs into zebrafish embryos. Chicken gicerin, under control of the neurofilament gene promoter, was preferentially expressed in neuronal cells and gicerin-expressing neurons exhibited a fasciculation formation with neighboring gicerin-positive axons, which may be partly due to homophilic cell adhesion activity of gicerin. These experimental results suggest that this fast and efficient transgenic animal system is useful for studying the functional roles of neuron-specific genes during the development.

KEY WORDS: Gicerin; neural cell adhesion molecule; transgenic zebrafish; functional analysis.

INTRODUCTION

Based on a variety of in vitro experiments and their patterns of expression in vivo, cell adhesion molecules have been implicated as playing major roles during the generation of neural specificity, including neurite outgrowth, growth cone guidance and target recognition (1). However, genetic analysis in both *Drosophila* and mouse has not yet completely evaluated these functions. Some advanced genetic approaches in *Drosophila* are providing a clue to understanding the in vivo roles of neural cell adhesion molecules. For example, ectopic expression of a member of immunoglobulin superfamily showed an altered pattern of growth cone guidance in certain neurons (2). Such approaches, however, are not always practical for the investigation of vertebrate molecules.

Transgenesis has been attempted in many vertebrates by injecting plasmid DNA into fertilized eggs, raising embryos to adults, breeding the offspring, and screening them for transmission of injected sequences. However, the long generation time of some animals makes this approach time-consuming and cumbersome. Furthermore, if the gene product to be expressed is fatal to the organism, it is impossible to establish stable transgenic animals.

Here we describe a convenient method for in vivo analyses of the roles of cell adhesion molecules in the vertebrate neural development, using transient transgenic

[1] Department of Pharmacology, Osaka University School of Medicine, Osaka, Japan.
[2] Department of Physiology, Keio University School of Medicine, Tokyo, Japan.
[3] National Institute for Physiological Sciences, Okazaki, Japan.
[4] Address reprint requests to: Dr. Naomasa Miki, Department of Pharmacology, Osaka University School of Medicine, 2-2 Yamadaoka, Suita, Osaka 565, Japan. Tel: 81-6-879-3520; Fax: 81-6-879-3529.
* Special issue dedicated to Dr. Kinya Kuriyama.

0364-3190/96/0200-0231$09.50/0

fish, that allows transgene expression in a directed pattern in normally developing nervous system. The zebrafish is well suited for the study of development, especially neuronal development. Hundreds of eggs can be produced daily on a year-round basis from a small number of adult fish. The rapid development of the virtually transparent embryos makes it possible to unambiguously identify many cell types as early as one day after fertilization. Recently, some approaches using transient transgenic zebrafish are providing a possibility of its usefulness in the analyses of mammalian genes, and their organization and expression pattern are highly conserved from fish to mammals (3,4,5). Our aim in the present study was to use this rapid and convenient zebrafish system to test the potential roles of chicken gicerin in neural development by directing its expression in the zebrafish nervous system.

In the previous studies, we have reported on a neurite outgrowth factor (NOF) and its receptor, gicerin, in chicken. Gicerin was first identified as a binding protein for NOF, a member of the laminin family of extracellular matrix proteins (6). Gicerin is an integral membrane glycoprotein of about 82 kD that is expressed temporally and spatially in the developing nervous system (7,8). By isolating and sequencing a gicerin cDNA, we have found that this protein is a novel member of the immunoglobulin superfamily. The in vitro experiments using L-cells transfected with gicerin cDNA showed that gicerin can function as a cell adhesion molecule which has both heterophilic and homophilic binding activities (9,10). These results lead to the suggestion that gicerin might play a role during axonal guidance, since they are expressed on both growth cones and axon pathways they follow.

For the further understanding of the in vivo roles of gicerin, we have generated transient transgenic animals by microinjecting chicken gicerin cDNA constructs into one-cell zebrafish embryos. The murine neurofilament promoter (NFP) was used to target the expression of transgene to the neural tissues. We investigated the expression pattern of gicerin in the developing zebrafish nervous system at cellular levels with whole-mount immunohistochemistry using a specific anti-gicerin antiserum.

EXPERIMENTAL PROCEDURE

Animals. Zebrafish were raised and maintained in aquaria at 28.5 °C on a 14h light/10h dark cycle essentially as described in the Zebrafish Book (11). Fertilized eggs were collected each morning and raised in an embryo-rearing solution (1/3 Ringer's solution, pH 7.2). Developmental stages (h) were expressed as hours after fertilization at 28.5 °C.

Recombinant DNA Constructions. All procedures were done following the standard recombinant DNA techniques (12). An expression vector pcDNA1 (Invitrogen) was digested with *AflII* and treated with T4 DNA polymerase to produce blunt ends and subsequently *HindIII* linkers were added. The resulting plasmid was inserted by a *XbaI* fragment of the *E. coli* β-galactosidase reporter gene (lacZ) at the *XbaI* site (13). Then the CMV promoter region was removed from this plasmid by digestion with *HindIII* and ligated to make a promoter-proving vector, containing lacZ reporter gene downstream from the *HindIII* site. To construct pNFP-lacZ, a 1.7-kb *HindIII* fragment containing the promoter and enhancer of the murine neurofilament gene (14) was inserted into the *HindIII* site of this plasmid. For the construction of pNFP-gicerin, pNFP-lacZ was digested with *XbaI*, religated, and then inserted by a *BamHI/EcoRI* fragment containing the full-length chicken gicerin cDNA sequence (9) at the comparable site of the plasmid (Fig. 1).

Microinjection and Detection of β-Galactosidase Activity. For injection, recently fertilized zebrafish eggs were pipetted onto an agarose ramp formed by resting glass slides in the lid of 100-mm Petri dish containing molten 1% agarose. Supercoiled plasmid DNA was injected into embryos prior to first cleavage with the aid of a micromanipulator. The injection solution contained 0.5% phenol red (used to estimate the volume of the injection) and 25 μg/ml DNA in 0.1 M KCl (15,16). To determine the patterns of β-galactosidase expression, embryos were fixed at 24 h and assayed for β-galactosidase activity essentially as described by Westerfield et al (3). Briefly, embryos were fixed in 4% paraformaldehyde, 4% sucrose, 0.15 mM $CaCl_2$, and 0.1 M sodium phosphate buffer (pH 7.2) for 1 hr at 4°C. The embryos were then rinsed in 0.1 M sodium phosphate, incubated in 1 mg/ml 5-bromo-4-chloro-indoyl-β-D-galactopyranoside (X-Gal; Stratagene), 150 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $K_4[Fe_2(CN)_6]$, 1.5 mM $K_3[Fe_2(CN)_6]$, in 5 mM sodium phosphate buffer (pH 7.2), at 37°C for 3 hours, fixed again, and then mounted in 50% glycerol and 0.1 M phosphate buffer. Individual transgene-positive cells were counted and assigned to specific tissues, according to their locations and morphologies. Embryos that were obviously malformed were excluded from the analyses.

Whole-Mount Immunohistochemistry. To provide a three-dimensional view of the distribution and morphology of gicerin-positive cells, we reacted whole embryos as following procedures. Embryos were dechorinated by treating with 1 mg/ml pronase for 1 min, rinsed several times, and fixed for 4 hr with 4% paraformaldehyde in 0.1 M phosphate buffer containing 0.15 mM $CaCl_2$ and 4% sucrose (pH 7.2). They were rinsed with 0.1 M phosphate buffer and then with distilled water, and permeabilized with acetone at −20°C for 7 min. After rinsing with distilled water, endogenous peroxidase was inactivated by treating the embryos with 0.3% hydrogen peroxide in methanol for 30 min. The embryos were rinsed with distilled water once more, then with 0.1 M phosphate buffer, and treated with 2% normal goat serum in phosphate-buffered saline (pH 7.2), 1% BSA, 1% DMSO and 0.1% Triton X-100 for 30 min to block non-specific binding sites. Following blocking, the embryo was incubated overnight at 4°C in the primary antibody, rinsed, incubated for 6 hr in biotinylated goat anti-rabbit secondary antibody, and then for 1 hr in horseradish peroxidase (HRP) avidin-biotin complexes (Vectastain Elite ABC kit, Vector Laboratories). The peroxidase reaction was carried out with diaminobenzidine (DAB) as the chromogen. To clear the embryos, they were immersed in a 1:2 mixture of benzyl alcohol and benzyl benzoate after dehydrating with 100% methanol. In the case of double-immunostaining, embryos were serially immunostained with anti-gicerin antiserum, alkaline phosphatase conjugated secondary antibody, and its substrate kit IV (BCIP/NBT,

Fig. 1. Recombinant DNA constructs used to generate transient transgenic zebrafish embryos. To direct expression of transgenes to the nervous system, the 1.7kb of the murine neurofilament gene promoter (NFP) was used. The construct NFP-lacZ contains the NFP (lightly striped boxes) fused to *E. coli* lacZ reporter gene (striped box), whereas the construct NFP-gicerin contains the NFP cloned in the front of the chicken gicerin cDNA (solid box). polyA (open boxes); simian virus 40 polyadenylation site.

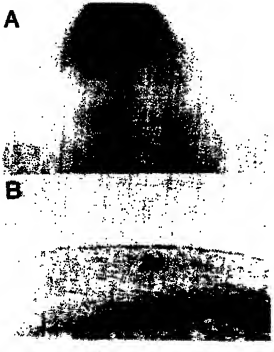


Fig. 2. The murine neurofilament gene promoter (NFP) directed the expression of a marker gene, β-galactosidase, to neuronal cells in transient transgenic zebrafish embryos at 24h. The β-galactosidase activity was detected by X-gal staining of the transgenic embryos in a whole-mount preparation. A, The NFP-lacZ transgene was activated in a trigeminal sensory ganglion neuron located caudal to the developing eyes (asterisks). Horizontal view, rostral is the bottom. B, A transgene-positive Rohon-Beard neuron in the dorsal region of spinal cord in a transgenic embryo. Materials of ventral region are yolk granules. Side view; rostral is to the right and dorsal is up.

Vector Laboratories) which develops into blue color. Then the embryos were double-stained with a monoclonal HNK-1 antibody which could stain almost developing neurons, next with a biotinylated goat anti-mouse IgM secondary antibody, and HRP staining as explained above. Unless otherwise stated, all incubations and rinses were carried out at room temperature on a shaker table. Individual expressing cells were counted and identified according to their locations and morphologies using Nomarski differential interference contrast microscopy.

RESULTS

Activation of Murine Neurofilament Gene Promoter in Zebrafish. Tissue-specific promoters of zebrafish itself are at present not available for the directed expression of transgenes. We first examined whether the activation of murine neurofilament gene promoter (NFP) was restricted to the zebrafish nervous system, as previously described for its expression in mice (14). The putative endogenous fish neurofilament proteins, which can be detected with antisera derived from the mammalian neurofilament proteins, is known to be expressed in the central and peripheral neurons (17, 18). We visualized the activity of NFP in zebrafish by microinjecting recombinant DNA constructs into embryos at the one-cell stage and then assaying β-galactosidase expression in the embryos after fixation at 24 h. Fig. 2 shows transient transgenic embryos in which the expression of lacZ transgene was directed to neuronal cells in the nervous system. The number of cells expressing the transgene was varied from 1 to 20 cells, dependent on individual embryos, probably due to an uneven, mosaic distribution of the injected DNA (3). The expression of NFP-lacZ was prominently detected in trigeminal ganglion neurons (Fig. 2A) and Rohon-Beard neurons (Fig. 2B), both of which are primary sensory neurons that mediate touch sensitivity. These neurons initiate axogenesis at the early time, and appear to be the first neurons in the zebrafish embryo (19). As an expression marker, we used a lacZ gene encoding β-galactosidase, the most commonly used enzymatic reporter molecule.

Since the β-galactosidase was not readily transported into axons in transgene-positive neurons, axons from the β-galactosidase expressing neurons were hardly stained (Fig. 2). We then examined the distribution of all transgene-positive cells which were classified into 5 different type of cells as described in the legend of Fig. 3, and counted 114 cells in 51 animals for NFP-lacZ, and 260 cells in 72 animals for NFP-gicerin. The percentage of transgene-positive cells was plotted against the classified cells in each category. We found that transient transgenic zebrafish embryos preferentially activated NFP in the nervous system. In respect to its expression level, these NFP-drived transgenes showed high levels of expression in neurons compared to that of the other type of cells, if any (data not shown). Our results suggest that the murine neurofilament gene promoter is functionally working in zebrafish and is activated within neurons as seen in transgenic mice. This indicates that transcription factors in zebrafish have an ability to recognize *cis*-acting elements of murine NF gene.

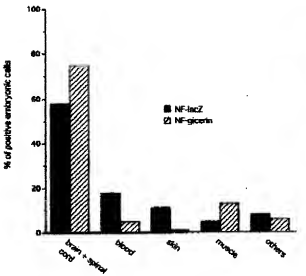


Fig. 3. Tissue distribution of cells expressing transgenes directed by the neurofilament promoter (NFP). The percentage of the total number of transgene-positive cells was plotted against 5 classified cell types where 114 cells in 51 animals were counted for NFP-lacZ and 260 cells in 72 animals for NFP-gicerin. Nervous system cells were identified on the basis of their locations within the brain or spinal cord. Blood cells were identified by their rounded morphologies and locations in the blood stream. Skin cells were the most superficial, flattened cells of the embryo. Muscle cells were identified by their elongated morphologies and striations. Cells that could not be placed into one of these four categories or that were ambiguous to identify were classified as others.

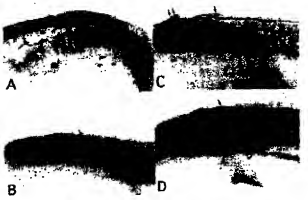


Fig. 4. Time course of gicerin expression under the control of neurofilament gene promoter. The NFP-gicerin transgene was detected in the developing neuronal cells around 20h of embryonal stage (A). Differentiating neuroepithelial cells (asterisks) in the hindbrain of a transgenic zebrafish embryo were stained weakly with the anti-gicerin antiserum. The cell bodies of transgene-positive neurons are indicated by arrows. Through 22h (B) and 24h (C and D), gicerin-expressing Rohon-Beard neurons projected their central axons bidirectionally and participated in the formation of the longitudinal axon tract in the spinal cord (arrowheads), while their peripheral axons (open arrowheads) branched in the skin. Peripheral axons of neurons in (C) are out of the plane of focus. In all panels anterior is to the left, and dorsal is upwards.

Neuron-Specific Expression of Chicken Gicerin in Zebrafish. We also used an NFP for the directed expression of chicken gicerin in the developing nervous system in zebrafish. Chicken gicerin was expressed exclusively in neuronal cells one day after transgene injection, when large numbers of neurons become postmitotic and extend neurites (19). No gicerin-positive endogenous materials were detected in normal zebrafish embryos when stained with an anti-chicken gicerin antiserum (n = 50, data not shown). As shown in Fig. 4, the expression of chicken gicerin in zebrafish was temporally and spatially regulated by the murine neurofilament gene promoter. The NFP-gicerin transgene was detected in the developing neuronal cells around 20h of embryonal stage when primary neurons differentiated and started to project their axons. On the beginning of differentiation, neuroepithelial cells in the hindbrain of a transgenic zebrafish embryo were stained weakly with an anti-gicerin antiserum (Fig. 4A). Through 22h and 24h of the embryonal development, gicerin-expressing Rohon-Beard neurons projected their central axons both anteriorly and posteriorly, and participated in the formation of the longitudinal axon tract in the spinal cord (Fig. 4B and C). The peripheral axons from a Rohon-Beard neuron formed an extensive arborization on the surface of body, as a general pattern of primary sensory neurons (Fig. 4D).

Examination of the immunostaining pattern in gicerin-positive neurons revealed a characteristic outlining of the somata of stained cells as well as complete staining of the cell processes, including the central and peripheral axons and their growth cones (Fig. 4 and 5). These cellular localization of gicerin in transgene-positive neurons in zebrafish brain were similar to that of gicerin-expressing neurons in the developing chick brain (unpublished data). In embryos containing a large number of cells expressing transgene, gicerin-positive neurons showed a fasciculation formation between their contacting central axons, resulting in a specific path of axonal tract, while their peripheral axons were in a form of nonfasciculated arborizations. A representative transgenic embryo was demonstrated in Fig. 5. The growth cones of central axons projected from two neighboring gicerin-positive Rohon-Beard neurons in the spinal cord exhibited a beginning of fasciculation formation, by catching and following the preceding axons each other (Fig. 5A). From the double-immunostaining of the same embryo with a HNK-1 monoclonal antibody, which

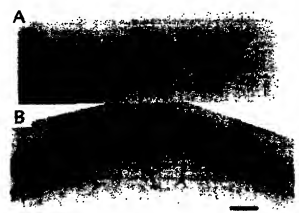


Fig. 5. Chicken gicerin was detected on the entire projection of axons as well as cell bodies in transgene-positive neurons in transgenic embryos stained with an anti-gicerin antiserum in a whole-mount preparation. As an example, two gicerin-expressing Rohon-Beard neurons (indicated as 1 and 2) in the spinal cord of a transgenic embryo were shown (A). They project central axons both anteriorly and posteriorly within the dorsolateral spinal cord, while their peripheral axons are arborizing in the surface of trunk. The growth cones of their central axons (arrowheads for axons projected from neuron 1 and arrows for axons from neuron 2) are catching and following the preceding axons at the sites of contact (asterisks) where they may start to fasciculate and form the longitudinal axon tract. In (B), the same embryo in (A) was double-stained with an anti-HNK-1 antibody which could stain almost developing neurons. Including the transgene-positive neurons, HNK-1 epitope-containing neurons are forming two bundles of dorsolateral axonal tracts within the developing spinal cord. Anterior is to the left, and dorsal is upwards. The scale of (A) is 90% of (B). Bar, 25 um.

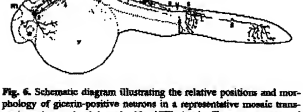


Fig. 6. Schematic diagram illustrating the relative positions and morphology of gicerin-positive neurons in a representative mosaic transgenic embryo microinjected with pNFP-gicerin. Twenty neurons and no non-neuronal cells were detected by an anti-gicerin antiserum in this embryo. Only one side of the body was illustrated for the simplicity. The transgene was expressed in various type of neurons in transgenic embryos at 26 h. Anti-gicerin antiserum stained a neuron (1) located in the region of ventrocaudal cluster in the brain, its long descending axon extended to the half of body length in path of the ventral longitudinal tract (arrow). In parallel with this axonal tract, descending central-axons of trigeminal neurons (2) and central axons of Rohon-Beard neurons (5) were arranged in the path of dorsal longitudinal axon tract (arrowhead), while their peripheral axons arborize extensively in the skin. Neurons in the hindbrain (3), in the region of posterior commissure (4) and others are also shown. m; midbrain, h; hindbrain, o; otic vesicle, e; eye, y; yolk.

could stain almost developing neurons, this fasciculation was confirmed as a longitudinal axon tract in the dorsolateral spinal cord (Fig. 5B). It is considered that the formation of a fasciculation between gicerin-positive neurons in transient transgenic zebrafish may partly result from the homophilic cell adhesion activity of exogeneous gicerin.

With the prominent expression of gicerin in the primary sensory neurons (trigeminal ganglion neurons and Rohon-Beard neurons), the transgene was also detected in various types of neurons in the transgenic zebrafish embryos according to their developmental stage; neurons in the region of the ventrocaudal cluster in the brain, hindbrain, otic vesicle, postotic commissure, motoneurons, ascending and descending interneurons, and so on. Among hundreds of gicerin-positive embryos, we illustrated a representative transgenic animal with schematic diagram which shows the relative position and morphology of gicerin-positive neurons (Fig. 6).

DISCUSSION

We firstly found that the murine neurofilament gene promoter is activated in specific regions of developing zebrafish embryos, suggesting that the promoter activity is conserved from fish to mammals. Previously, several groups have studied NFP-deriven reporter genes in different mammalian cell lines, but no cell-type specific expression was observed (14). These results imply that in vitro expression experiments of NF gene do not address the tissue-specificity of NF gene expression. Therefore, experiments were required using transgenic animal in order to examine the neural specificity of NF gene expression (20, 21). Our data using zebrafish are comparable to those of transgenic mice, with regard to the tissue-specificity of NF gene promoter, and support the usefulness of this rapid transient transgenic animal system in the functional analyses of promoter activities that regulate gene expression in vertebrates.

In this transient expression system, the majority of animals contained a small number of transgene-positive cells per embryo, probably due to a mosaicism of transgene expression (3) or its combination with tissue-specificity of the promoter. The restricted pattern of transgene expression, however, is rather favorable for the analyses of the roles of cell adhesion molecules in the developing nervous system, because the expression of transgene only in a few cells is easy to follow overall development of the neurons in transgenic embryos.

We used the NFP to direct ectopic expression of chicken gicerin to the nervous system in zebrafish. Tran-

sient transgenic embryos injected with the NFP-gicerin construct showed somewhat more neuron-specific pattern of expression than those injected with NFP-lacZ construct. This is probably due to difference in sensitivity of the assays used to detect transgene expression or promoter strength between nervous system and the other tissues. The other possibility is that the expression of chicken gicerin affected cell differentiation of the developing zebrafish embryos. It has been reported that a switch in cell fate can be induced by ectopic expression of a gene (22,23).

Chicken gicerin was expressed on the entire projections of neurons, including axons and growth cones as well as cell bodies, in transgenic embryos. Axons from gicerin-expressing zebrafish neuronal cells exibited a fasciculation formation with neighboring gicerin-positive axons, may partly due to homophilic cell adhesion activity of gicerin (9,10). Although we did not examine the interactions between chicken gicerin and endogenous molecules in zebrafish, it is possible that the presence of NOF- and gicerin-like molecules in zebrafish can interact with the exogenous chicken gicerin by heterophilic or homophilic cell adhesive activities. Recently, several studies have provided new insights into the cellular and molecular cues required for correct axonal pathfinding (24,25,26). These studies have uncovered the intriguing evolutionary conservation of the basic molecular and cellular mechanisms underlying growth cone guidance and target recognition, since many proteins involved in such process share structural similarities in different species.

In conclusion, our experimental results support the usefulness of the transient transgenic system using zebrafish in the analyses of gene promoter activity, and suggest that transgenic zebrafish system may be very useful in studying the in vivo function of a variety of genes during neural development.

REFERENCES

1. Doherty, P., and Walsh, F. S. 1994. Signal transduction events underlying neurite outgrowth stimulated by cell adhesion molecules. Curr. Opin. Neurobiol. 4:49–55.
2. Lin, D. M. and Goodman, C. S. 1994. Ectopic and increased expression of Fasciclin II alters motoneuron growth cone guidance. Neuron 13:507–523.
3. Westerfield, M., Wegner, J., Jegalian, B. G., DeRobertis, E. M., and Puschel, A. W. 1992. Specific activation of mammalian Hox promoters in mosaic transgenic zebrafish. Gene & Dev. 6:591–598.
4. Reinhard, E., Nedivi, E., Wegner, J., Skene, J. H. P., and Westerfield, M. 1994. Neural selective activation and temporal regulation of a mammalian GAP-43 promoter in zebrafish. Development 120:1767–1775.
5. Kuo, C-H., Uetsuki, T., Kim, C. H., Tanaka, H., Li, B-S., Taira, E., Higuchi, H., Okamoto, H., Yoshikawa, K., and Miki, N. 1995. Determination of a neo3in cis-acting element required for neuron specific expression by using zebrafish. Biochem. Biophys. Res. Comm. 211:438–446.
6. Hayashi, Y. and Miki, N. 1985. Purification and characterization of a neurite outgrowth factor from chicken gizzard smooth muscle. J. Biol. Chem. 260:14269–14278.
7. Taniura, H., Kuo, C-H., Hayashi, Y., and Miki, N. 1991. Purification and characterization of an 82-kD membrane protein as a neurite outgrowth factor binding protein: possible involvement of NOF binding protein in axonal outgrowth in developing retina. J. Cell. Biol. 112:313–322.
8. Kuno, S., Taniura, H., Taira, E., and Miki, N. 1992. Involvement of a receptor for neurite outgrowth factor (NOFR) in cerebellar neurogenesis. Neurosci. Lett. 140:78–80.
9. Taira, E., Takaha, N., Taniura, H., Kim, C-H., and Miki, N. 1994. Molecular cloning and functional expression of gicerin, a novel cell adhesion molecule that binds to neurite outgrowth factor. Neuron 12:861–872.
10. Taira, E., Nagino, T., Taniura, H., Takaha, N., Kim, C-H. Kuo, C-H., Li, B-S., Higuchi, H., and Miki, N. 1995. Expression and functional analysis of a novel isoform of gicerin, an immunoglobulin superfamily cell adhesion molecule. J. Biol. Chem. 270: 28681–28687.
11. Westerfield, M. 1993. The Zebrafish Book, 2nd edition. University of Oregon Press.
12. Maniatis, T., Frisch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
13. Hall, C. V., Jacob, P. E., Ringold, G. M., and Lee, F. 1983. Expression and regulation of *Escherichia coli lacZ* gene fusions in mammalian cells. J. Mol. Appl. Genet. 2:101–109.
14. Nakshira, K., Ikenaka, K., Wada, K., Tamura, K., Furuichi, T., and Mikoshiba, K. 1990. Structure of the 68-kDa neurofilament gene and regulation of its expression. J. Biol. Chem. 265:19786–19791.
15. Culp, P., Nüsslein-Volhard, C., and Hopkins, N. 1991. High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. Proc. Natl. Acad. Sci. USA 88:7953–7957.
16. Stuart, G. W., Vielkind, J. R., Mcmurray, J. V., and Westerfield, M. 1990. Stable lines of transgenic zebrafish exhibit reproducible patterns of transgene expression. Development 109:577–584.
17. Mencarelli, C., Magi, B., Marzocchi, B., Contorni, M., and Pallini, V. 1991. Evolutionary trends of neurofilament proteins in fish. Comp. Biochem. Physiol. 100B:733–740.
18. Ishikawa, Y. 1990. Development of muscle nerve in the teleost fish, medaka. Neuroscience Research, Suppl. 13:S152–S156.
19. Metcalfe, W. K., Myers, P. Z., Trevarrow, B., Bass, M. B., and Kimmel, C. B. 1990. Primary neurons that express the L2/HNK-1 carbohydrate during early development in the zebrafish. Development 110:491–504.
20. Begemann, M., Tan, S-S., Cunningham, B. A., and Edelman, G. M. 1990. Expression of chicken liver cell adhesion molecule fusion genes in transgenic mice. Proc. Natl. Acad. Sci. USA 87: 9042–9046.
21. Byrne, G. W., and Ruddle, F. H. 1989. Multiplex gene regulation: A two-tiered approach to transgene regulation in transgenic mice. Proc. Natl. Acad. Sci. USA 86:5473–5477.
22. Van Vactor, D. L., Cagan, R. L., Kramer, H. and Zipursky, S. L. 1991. Induction in the developing compound eye of Drosophila: Multiple mechanisms restrict R7 induction to a single retinal precursor cell. Cell 67:1145–1155.
23. Dorsky, R. I., Rapaport, D. H., and Harris, W. A. 1995. Xotch inhibits cell differentiation in the Xenopus retina. Neuron 14:487–496.

24. Kanki, J. P., Chang, S., and Kuwada, J. Y. 1994. The molecular cloning and characterization of potential chick DM-GRASP homologs in zebrafish and mouse. J. Neurobiol. 25:831–845.
25. Ramos, R. G., Igloi, G. L., Lichte, B., Baumann, U., Maier, D., Schneider, T., Brandstatter, J. H., Frohlich, A., and Fischbach, K. F. 1993. The irregular chiasm C-roughest locus of Drosophila, which affects axonal projections and programmed cell death, encodes a novel immunoglobulin-like protein. Genes Dev. 7:2533–2547.
26. Grenningloh, G., Bieber, A. J., Rehm, E. J., Snow, P. M., Traquina, Z. R., Horrach, M., Patel, N. H., and Goodman, C. S. 1990. Molecular genetics of neuronal recognition in *Drosophila*: Evolution and function of immunoglobulin superfamily cell adhesion molecules. Cold Spring Harbor Symp. Quant. Biol. 55:327–340.

EXHIBIT 9

Gene, 173 (1996) 89–98

GENE 09432

Green fluorescent protein marks skeletal muscle in murine cell lines and zebrafish *

(Transfection: *Danio rerio*; embryo; myosin light chain; vital dye)

Jennifer Barnett Moss, Alivia L. Price, Erez Raz, Wolfgang Driever and Nadia Rosenthal

Cardiovascular Research Center, Massachusetts General Hospital East, Charlestown, MA 02129, USA

Received by D.C. Youvan: 1 May 1995; Revised/Accepted: 25 August 1995; Received at publishers: 9 October 1995

SUMMARY

The green fluorescent protein (GFP) acts as a vital dye upon the absorption of blue light. When the *gfp* gene is expressed in bacteria, flies or nematodes, green fluorescence can be directly observed in the living organism. We inserted the cDNA encoding this 238-amino-acid (aa) jellyfish protein into an expression vector containing the rat myosin light-chain enhancer (MLC-GFP) to evaluate its ability to serve as a muscle-specific marker. Transiently, as well as stably, transfected C2C12 cell lines produced high levels of GFP distributed homogeneously throughout the cytoplasm and was not toxic through several cell passages. Expression of *MLC-GFP* was strictly muscle-specific, since Cos 7 fibroblasts transfected with *MLC-GFP* did not fluoresce. When GFP and βGal markers were compared, the GFP signal was visible in the cytoplasm of the living cell, whereas visualization of βGal required fixation and resulted in deformation of the cells. When the *MLC-GFP* construct was injected into zebrafish embryos, muscle-specific *gfp* expression was apparent within 24 h of development. *gfp* expression was never observed in non-muscle tissues using the *MLC-GFP* construct. Transgenic fish continued to express high levels of *gfp* in skeletal muscle at 1.5 months, demonstrating that GFP is an effective marker of muscle cells in vivo.

INTRODUCTION

The beautiful green bioluminescence emanating from the circular and radial food canals of the jellyfish *Aequoria victoria* is ultimately caused by the emission of green light from a cyclical amino-acid (aa) chromophore which is an integral part of the green fluorescent protein (GFP) (Prasher et al., 1992). When *gfp* cDNA was cloned and expressed in bacteria, *Caenorhabditis elegans* (Chalfie et al., 1994) or in *Drosophila melanogaster* (Wang et al., 1994), a green fluorescence (GF), with an emission peak at 509 nm, was directly visualized in vivo. Many reporter systems allow the quantitation and localization of proteins expressed within prokaryotic as well as eukaryotic cells. A distinct advantage of GFP is that its fluorescence depends only upon the presence of the protein (inducible at blue excitation wavelengths in the 360–420 nm range). Therefore, GFP is a marker whereby GF can be plotted

Correspondence to: Dr. J.B. Moss, CVRC, MGH-E, 149 13th Street, Mailcode 1494201, Charlestown, MA 02129, USA. Tel. (1-617) 724-9562; Fax (1-617) 724-9561; e-mail: moss@helix.mgh.harvard.edu

* Presented at the Palo Alto Institute of Molecular Medicine Symposium on 'Fluorescent Proteins and Applications', Hyatt Rickeys, Palo Alto, CA, USA, 6–7 March 1995.

Abbreviations: *A., Aequoria*: aa, amino acid(s); βGal, β-galactosidase (product of gene *lacZ*); bp, base pair(s); *C., Caenorhabditis*: CAT, chloramphenicol acetyltransferase; cDNA, DNA complementary to RNA; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; dNTP, deoxyribonucleotide triphosphate; *D., Drosophila*; FCS, fetal calf serum; FITC, fluoroisothiocyanate; GF, green fluorescence; GFP, GF protein; *gfp*, gene (DNA) encoding GFP; Hy, hygromycin; kb, kilobase(s) or 1000 bp; MLC, myosin light chain; *MLC*, gene encoding MLC; *MLCE*, *MLC* element; nt, nucleotide(s); PCR, polymerase chain reaction; *p*, promoter; p, plasmid; Polfk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistant/resistance; S65T, Ser^{65} → Thr; *UTR*, untranslated region(s); wt, wild type.

SSDI 0378-1119(95)00729-6

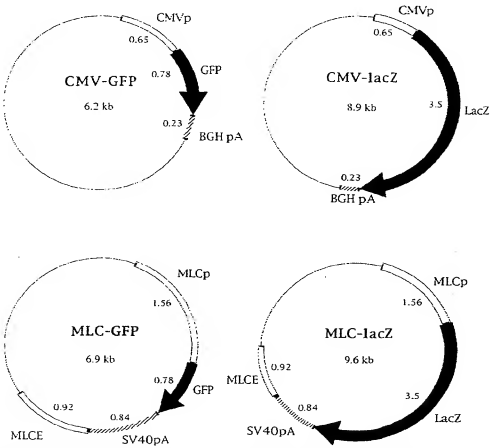
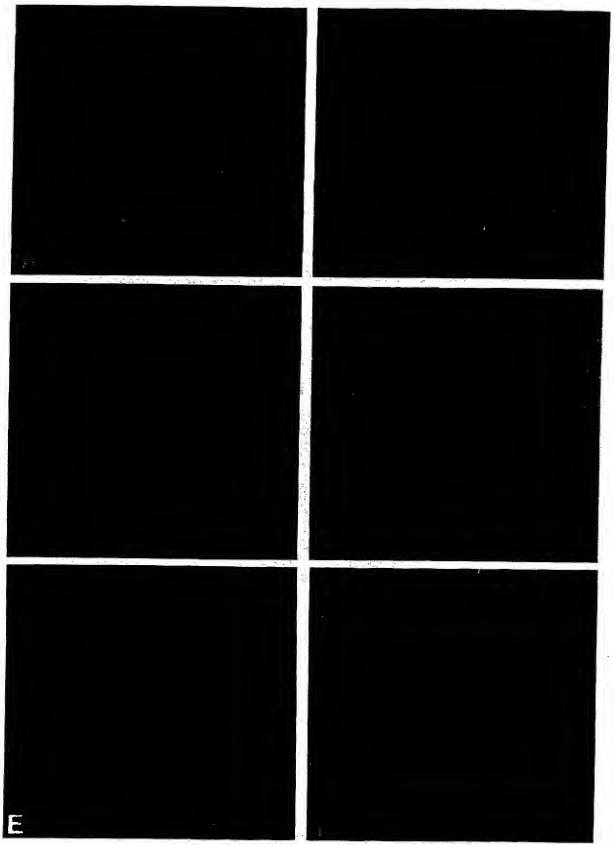


Fig. 1. The *gfp* plasmid expression vectors were derived from TUβ65, a Bluescript (Stratagene, La Jolla, CA, USA) vector containing the full-length cDNA fragment from *A. victoria* (Chalfie et al., 1994). A rat *MLC* expression vector (Donoghue et al., 1988) with a unique *Hind*III site at the junction between the 1.5-kb *MLC* promoter and the 0.8-kb SV40 poly(A) sequence fused to the muscle specific *MLC* 0.9-kb enhancer was linearized, then blunt ended using Pol1k and dNTPs (Ausubel et al., 1993). A micrococcal-nuclease-treated 0.78-kb *Kpn*I-*Eco*R1 *gfp* fragment from TUβ65 was subsequently ligated into the *MLC* vector to create *MLC-GFP*. CMV vectors were constructed in pcDNA3 (Invitrogen). The *Kpn*I-*Eco*R1 *gfp* cDNA was directionally cloned into the corresponding sites of the pcDNA3 polylinker (*CMV-GFP*). The *lacZ* expression vectors were constructed with a 3.5-kb *Not*I fragment of an SV40 *lacZ* vector (pSVβ, MacGregor and Caskey, 1989) inserted into the *Not*I site of pcDNA3 to yield *CMV-lacZ*, or blunt end ligated into the *MLC-GFP* vector described above, to generate *MLC-lacZ*. A serine within the chromophore was mutated to a Thr (TCT to ACT) by a modified PCR mutagenesis (Innis et al., 1990). The mutation was confirmed by DNA sequencing and the mutated *gfp* was cloned into pcDNA3 for comparison to wt.

Fig. 2. Applications of GFP. (**A**) Live cultures of Cos 7 cells transfected with *CMV-GFP* (10× lens). (**B**) Background fluorescence of untransfected Cos 7 cells (10× lens). (**C**) Bright field photograph of growing Cos 7 fibroblasts. (**D**) Cos 7 cells transfected with *CMV-lacZ* then fixed after 48 h in culture (20× lens). βGal activity was visualized as previously described (Ausubel et al., 1993). (**E**) Live Cos 7 cell cultures transfected with *CMV-GFP* containing the S65T mutation (25× water immersion lens). (**F**) Live Cos 7 cells transfected with wt *CMV-GFP* photographed under the same conditions as for **E. Methods:** The indicated plasmids were CsCl gradient purified then transiently transfected into Cos 7 fibroblast cell lines. 60 mm culture dishes containing 5×10^5 cells were incubated for 8 h with 3 μg DNA/10 μl lipofectamine (Gibco BRL)/3 ml DMEM. The transfection media was removed and replaced with culture medium (DMEM with 10% FCS/100 units streptomycin/100 μg penicillin/ml) for 14–20 h. GF was visualized using an Optiquip model #1500 power supply with a Xe/Hg light source set at 200 W (Hg lines). Photographs were taken on 400 speed film through a Zeiss axioplan microscope. The filter set was either a Zeiss #10 (FITC) or a customized set which eliminates some of the green autofluorescence (Chroma standard GFP set).


E

over time in living cells or transparent organisms with no addition of exogenous substrates. Bacteria engineered with a T7 RNA polymerase promoter generate high levels of GFP (Chalfie et al., 1994; Inouye et al., 1994) that can be aerobically cultured without loss of GF or toxicity to the cells. In *C. elegans*, the β-tubulin promoter *mec-7* produces bright green touch-receptor neurons that can be observed continuously during development in the same animal (Chalfie et al., 1994). When a maternally-derived GFP fusion protein is expressed in transgenic *D. melanogaster*, the fluorescent oocytes are resistant to photobleaching, are homogeneously labeled and can be directly observed with the confocal microscope in living, developing egg chambers (Wang et al., 1994). Other applications for GFP as a vital dye are just beginning to be realized.

Because GFP is a relatively small protein that fluoresces without the need for additional accessory molecules we evaluated its potential for tissue-specific expression during muscle development. The availability of muscle-specific control regions with known developmental profiles, such as the promoter and enhancer from the rat myosin light chain 1/3 locus, provide the opportunity to produce GFP-marked muscle cells which can be followed over time. The results of this report indicate that GF is readily visible in murine C2C12 muscle cells as transient and stable transfectants. Skeletal muscle fibers in transient transgenic zebrafish embryos and adults can be marked with GFP and observed with a fluorescent microscope in living animals. Expression is tissue specific, durable over time and non-toxic to the cell types we have studied. Muscle-specific GFP markers and different stage-specific control regions will provide powerful new tools for the study of zebrafish muscle development.

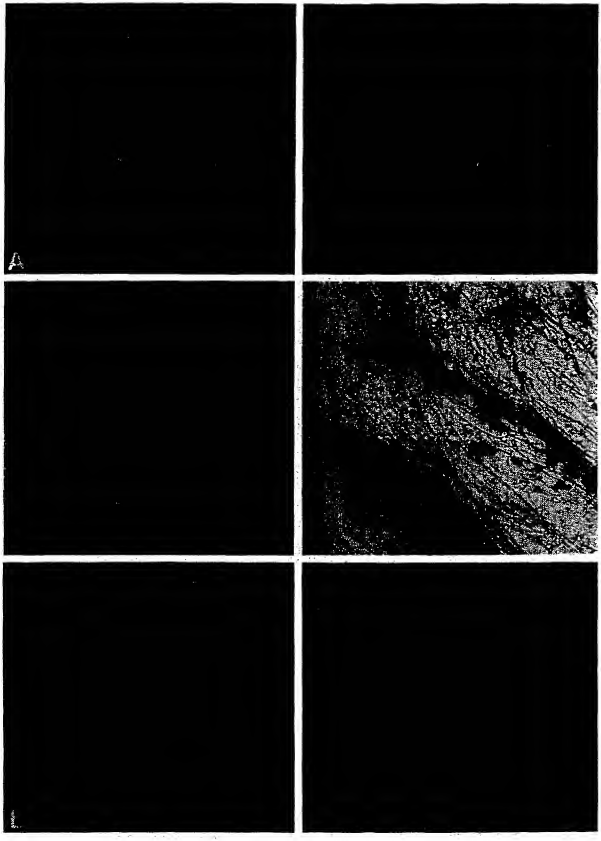
RESULTS AND DISCUSSION

(a) GFP is expressed transiently in Cos 7 fibroblasts and C2C12 myotubes

We determined if GFP could be used as a vital dye in transiently transfected cell lines by comparison with GFP and βGal signals. We placed the *gfp* cDNA from plasmid TU#65 (Chalfie et al., 1994) into two plasmids, one that was designed to function only in muscle cells (*MLC-GFP*) and one that could potentially be expressed in a variety of cell types (*CMV-GFP*). *MLC-GFP* (Fig. 1) is a pUC18-based myosin light chain vector consisting of 1.56 kb of the *MLC1* promoter, 0.84 kb of the small intron and polyadenylation site of the SV40 T antigen and 0.92 kb of the *MLC* enhancer (Donoghue et al., 1988). In transgenic mice, the *MLC* enhancer was sufficient to activate high levels of developmentally regulated gene expression from the *MLC1* promoter (Rosenthal et al., 1989). Further analysis of transgenic fetal and neonatal mice revealed that graded synthesis of CAT produced from this construct is established during the initial stages of somitogenesis and is maintained during subsequent maturation of somitic derivatives, persisting only in adult intercostal and intervertebral muscle groups (Donoghue et al., 1991; Grieshammer et al., 1992). In this study, the *MLC-GFP* construct lacked 5′ and 3′ *UTR* of *gfp*. The native jellyfish ATG, which is not a Kozak consensus sequence, was used for translation. For purposes of comparison, we inserted a *lacZ* reporter into the same site of a parallel *MLC* vector. We also generated *gfp* and *lacZ* mammalian expression vectors using the Invitrogen pcDNA3 plasmid (Fig. 1). This construct drives high-level stable and transient expression of genes inserted next to a *CMV* promoter.

When Cos 7 cells were transfected with *CMV-GFP*, bright GF was readily observable at 24 h post-transfection with a 10× lens using the standard GFP filter set from Chroma (Fig. 2A). Because this construct allows for episomal replication in Cos 7 cells which latently express the SV40 large T antigen, large amounts of GFP protein become concentrated in the cytoplasm. Positive cells were visualized without changing the media or perturbing the culture. Green auto-fluorescing dead cells which interfered with the GFP signal were removed by washing with phosphate buffered saline. Any remaining dead cells fluoresced under both GFP and Texas red filter sets, whereas the GF from GFP-positive cells was visible only with a GFP or FITC (fluoroisothiocyanate)

Fig. 3. GF of myotubes. (**A**) Live cultures of C2C12 cells transfected with *MLC-GFP* (25× lens, water immersion). (**B**) Background fluorescence of untransfected myotubes (25×). (**C**) Bright field image of myotubes in culture (25×). (**D**) C2C12 cells transfected with *MLC-lacZ* and fixed (20× lens). Panels **E** and **F**: two separate isolates of stable C2C12 myotubes. **Methods:** C2C12 myoblasts were seeded at 5×10^5 cells/60 mm dish in growth medium (DMEM/20% FCS/100 units streptomycin/100 μg penicillin/ml). Each lipofectamine transfection contained 20 μl lipofectamine and 5 μg DNA. The cells were incubated in growth media for 24 h, then differentiated to myotubes in low serum with DMEM/2% horse serum for an additional 48 h. *MLC-GFP* stable C2C12 cell lines were generated using a co-transfected Hy^R vector (p3′SS, Stratagene). 100-mm dishes were seeded with 0.1×10^6 myoblasts and lipofectamine-transfected using 15 μg of *MLC-GFP* and 1 μg of p3′SS in 6 ml of DMEM containing 30 μl lipofectamine. After 8 h in transfection media, cells were washed and replaced with growth media for 16 h. We generated a killing curve using Hy concentrations ranging from 200 to 800 μg/ml in growth media. We conclude that 600 μg Hy/ml is optimal for selection in C2C12 cells. The cells were incubated in selection media for one week and single colonies cloned and expanded. A portion of each clone was grown in low serum and tested for green fluorescence. Four of six Hy resistant lines resulted in bright green fluorescence upon differentiation into myotubes.


A

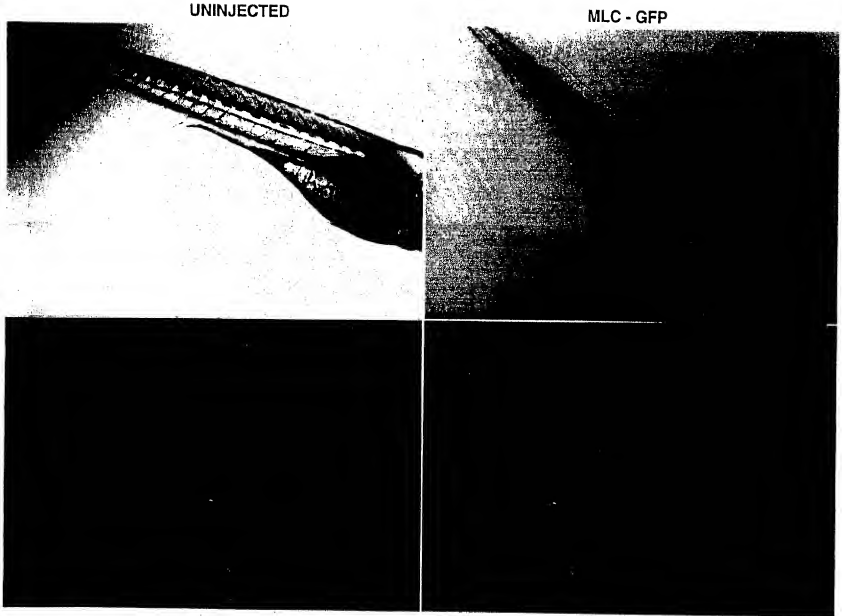


Fig. 4. Bright-field photograph and fluorescence image (below) of the same 36 h uninjected embryo (**left**) (pale green auto-fluorescence accumulated in the yolk sac) and of a 36 h *MLC-GFP* injected zebrafish (**right**). **Methods:** Zebrafish (*Danio rerio*) were mated and freshly fertilized eggs dechorionated with 5 μg pronase/ml. Embryos at the 2-, 4- or 8-cell stage were subsequently injected with 50 ng/μl of *MLC-GFP* or *CMV-GFP* plasmid DNA and 0.1% phenol red (Gibco BRL) as a marker. After 24 h of development in embryo media on agarose-coated culture dishes (Westerfield, 1993), the GFP was first visibly expressed in embryonic skeletal muscle fibers. All photographs were taken with a 5 × lens. Fish were maintained in the system as previously described (Solnica-Krezel et al., 1994). For photography, zebrafish embryos were placed in a depression slide, anesthetized and positioned in methyl cellulose. Once revived in egg water, embryos were returned to the system.

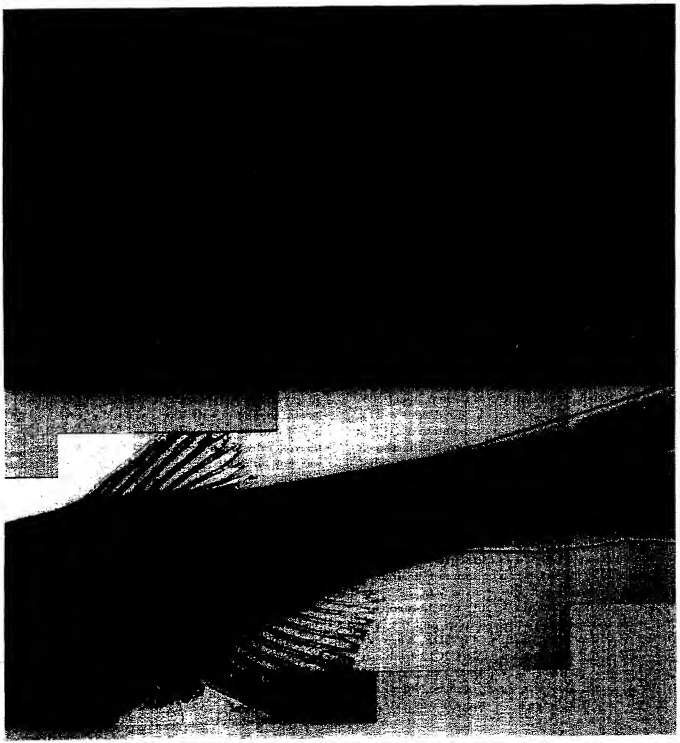


Fig. 5. Fluorescence (top) and bright field (bottom) photograph of a zebrafish at 1.5 months of development using a 5× lens Although the adult skeleton has formed and green auto-fluorescence accumulates in the swim bladder, this transiently transgenic zebrafish was still transparent to the wavelengths of light required to excite the GFP generated from the *MLC-GFP* plasmid.

filter set. *MLC-GFP* transfected into Cos 7 cells produced no GF (data not shown). The background auto-fluorescence from untransfected cells is shown in Fig. 2B. A bright field image (10× lens) depicts the growing Cos 7 cells (Fig. 2C). In Fig. 2D, Cos 7 cells were transiently transfected with the *CMV-lacZ* vector and the βGal

activity visualized after fixation (Ausubel et al., 1993). Although βGal can be visualized in the Cos 7 fibroblasts, these cells were fixed and treated with exogenous substrate, whereas endogenous GFP (Fig. 1A) was observed with the fluorescence microscope in living cells.

It has recently been reported that a Ser→Thr (S65T) mutation in the GFP chromophore improves GF in bacteria (Heim et al., 1995). We sought to determine if this mutation would fluoresce more brightly than wt GFP in Cos 7 cells. We generated an S65T mutation via PCR mutagenesis which was sequenced then subcloned into the pcDNA3 vector. Nominal differences in GF were observed between the S65T mutation (Fig. 2E) and the wt *CMV-GFP* construct (Fig. 2F) when transiently transfected into Cos 7 cells. Although we did not directly quantitate the fluorescence, we estimate the amount of GF resulting from the S65T mutation to be no more than 2-fold that of the *CMV-GFP* generated GF.

A parallel set of transfections was performed in C2C12 myoblasts (Fig. 3). The *gfp* expression from the *MLC-GFP* construct was evident only after 48 h in differentiation media (Fig. 3A). During this time, transfected C2C12 mono-nucleated myoblasts fused to form myotubes containing several nuclei within a single cytoplasm. GFP was produced throughout the entire cytoplasm in about 10% of the myotubes and was best visualized with a 25× water immersion lens. The reduced resolution of GFP in myotubes when compared with the Cos 7 cells may be due to the larger amount of cytoplasm in the myotubes, to the relative number of *gfp* transcripts produced by the two constructs, or to different translational or post-translational environments in the two cell types. In duplicate transient transfections of C2C12 cells, variability in the GF of different myotubes was also evident (data not shown). The background fluorescence of an untransfected culture of C2C12 myotubes can be seen in Fig. 3B along with a bright field image in Fig. 3C. The *MLC-lacZ* construct was separately transfected into C2C12 myoblasts which were differentiated into myotubes and the βGal activity visualized after fixation (Fig. 3D). When the *CMV-GFP* vector was transfected into C2C12 myoblasts, expression was apparent in dividing cells at about 12 h post-transfection. However, once the cells were differentiated into myotubes in low serum, GF was no longer apparent (data not shown).

(b) Stable C2C12 myotube cultures produce GFP

We determined whether *gfp* would continue to be expressed in mammalian cells when stably integrated into the chromosomes of muscle cells. C2C12 myoblasts were co-transfected in a ratio of 15:1 with the *MLC-GFP* construct and a Hy^R vector, respectively. Six resistant lines were expanded at low density as myoblasts. A portion of each clone was differentiated into myotubes and evaluated for GF. As shown in Fig. 3E and F, myotubes from two differentiated C2C12 stable cell lines displayed variable levels of *gfp* expression. Since we have not evaluated other stably transfected cell types, it is not clear whether the variability in GFP produced was due to myotube formation or is an inherent quality of the protein or its expression. The gene appeared to be stably transmitted and expressed since the cells still fluoresced after three passages in selection media (data not shown).

(c) Transiently transgenic zebrafish produce GFP

We employed the chordate fish *Danio rerio* (zebrafish) as a facile test for expression of the *MLC-GFP* construct in living animals. Because these fish are initially transparent, GF could be assayed directly using the same conditions as for the tissue culture cells. Non-linearized *MLC-GFP* plasmid was injected into fertilized zebrafish eggs and muscle-restricted GF was first observed in mosaic animals after 24 h of development. At this time, the embryos have just straightened and are beginning to flex their tail muscles. The injected embryo in Fig. 4 has developed approx. 30 somites. Its appearance under bright field microscopy can be viewed in Fig. 4. Of more than 50 expressing survivors, all had differing numbers of labeled somitic muscle fibers. The uninjected 36-h embryo in Fig. 4 produced background fluorescence primarily in the yolk cells. GF derived from the *MLC-GFP* construct was not observed in any other tissue of these mosaic animals. This observation is in contrast to embryos injected with the *CMV-GFP* construct where groups of unidentified, non-muscle cells at the ventral border of the yolk sac were labeled with GFP (data not shown).

One of the *MLC-GFP* injected survivors was observed with GF and bright field optics as a fry after 1.5 months of development (Fig. 5). Despite the development of scales, this older zebrafish continued to be transparent to the blue excitation wavelengths necessary to stimulate the visible muscle-specific GF observed in this transient transgenic animal. The *gfp* gene continued to be expressed and/or the protein was stabilized only in skeletal muscle cells.

(d) Conclusions

(*1*) In this study we have demonstrated the use of GFP as a vital dye in muscle cell cultures and transient transgenic zebrafish muscle. The primary advantage of GFP over other available reporters such as *lacZ* is that gene expression can be continuously observed in living cells, while fixed cells are lost to further analysis. Although it is possible to visualize βGal activity in living cells using the substrate fluorescein-di-β-D-galactopyranoside (Molecular Probes), *gfp*-expressing cells can be

observed directly without perturbing the cultures or animals with additional components. We envision that stable cell lines could be rapidly produced using GFP, since GF cells could be identified without drug selection.

(2) Since the GF gencrated in myotubes by the *MLC-GFP* construct was visible only at a 25-fold magnification or more, this suggested that the protein concentration was lower in these cells than in the *CMV-GFP* transfected Cos 7 cells where GF was visiblc using a 10× lens. This observation may be due to a combination of a weaker promoter and/or a larger cytoplasm when compared to the Cos 7 transfections. In addition, the expression or post-translational modification of GFP protein, as well as the redox environment could be more favorable in Cos 7 cells. Since muscle cells fuse to form syncytia of many nuclei contained within a single cytoplasm, a relatively strong muscle-specific promoter/enhancer construct such as *MLC-GFP* was capable of producing detectable levels of GFP protein in transient transfections. A weaker 1565-bp myogenin promoter (Cheng et al., 1993) regulating *gfp* expression generated very low levels of GF in transiently transfected C2C12 cells. However, when the *myogenin-GFP* plasmid was injected into zebrafish embryos, muscle-specific GF comparable to that generated by the *MLC-GFP* construct was detected after 24 h (data not shown). Perhaps the construct was integrated into the genome of the embryo such that multiple copies of *myogenin-gfp* were generated during the rapid replication of DNA before the midblastula transition when zygotic genes are transcribed (Westerfield, 1993). This would allow for the production of larger amounts of GFP protein than in transient transfections.

(3) The GFP protein is not toxic to mammalian cells since C2C12 cell lines expressing *gfp* can be passaged several times. Similarly, zebrafish embryos injected with the *MLC-GFP* construct transiently expressed high levels of tissue-specific protein without any deleterious effect. This property of GFP makes it a useful vital marker for the preparation of transgenic animals. Since the introduction of transgenes is very inefficient in zebrafish (Lin et al., 1994), a readily detectable marker would be invaluable. In addition, constructs carrying different promoters regulating *gfp* expression in a cell or tissue-specific manner could be used to follow developmental processes in vivo. Cell migration and morphogenesis could be directly compared in wt and mutant animals.

(4) The *MLC-GFP* stable cell lines generated GF only in myotubes, providing a useful marker for the study of muscle differentiation in cell culture. The timing of other muscle-specific regulatory regions expressing *gfp* could also be assayed in this system. We could not select *gfp*-expressing muscle cells directly since the *MLC* control regions initiate expression only during differentiation, when cells enter G_0 and no longer replicate. Although other constructs such as *CMV-GFP* may be expressed in a number of different zebrafish tissues, the *MLC-GFP* construct can be used in studies of muscle differentiation, since its expression is strictly muscle-specific. One disadvantage of the rat *MLC-GFP* construct is that it is not expressed until relatively late during development in the transiently transgenic zebrafish. Additional zebrafish-specific control elements may be required for accurate timing of expression. Notably, the rat MLC transcriptional control regions present in our construct were capable of directing muscle-specific expression of *gfp* in the zebrafish. This functional conservation of promoter and enhancer activity probably extends to other genes as well. We are currently investigating the use of other GFP muscle markers in the study of muscle differentiation in the developing zebrafish embryo.

ACKNOWLEDGEMENTS

We are grateful for helpful discussions with Mark Fishman. This work was supported by grants to N.R. from the NIH (RO1AR41926 and RO1AG12316) and a sponsored research agreement from Bristol-Myers Squibb. N.R. is an Established Investigator of the American Heart Association. Funding for J.B.M. is through the Muscular Dystrophy Association and NIH postdoctoral fellowship F32HD07926. E.R. is supported by a Human Frontier Science Program postdoctoral fellowship. Part of the work was supported by NSF IBN-9317469 to W.D.

REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Struhl, K. and Smith, J.A. (Eds.): Current Protocols in Molecular Biology. Wiley, New York, NY, 1993.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C.: Green fluorescent protein as a marker for gene expression. Science 263 (1994) 802–805.

Cheng, T.-C., Wallace, M.C., Merlie, J.P. and Olson, E.N.: Separable regulatory elements governing myogenin transcription in mouse embryogenesis. Science 261 (1993) 215–218.

Donoghue, M., Ernst, H., Wentworth, B., Nadal-Ginard, B. and Rosenthal, N.: A muscle-specific enhancer is located at the 3′ end of the myosin light-chain 1/3 gene locus. Genes Dev. 2 (1988) 1779–1790.

Donoghue, M., Merlie, J.P., Rosenthal, N. and Sanes, J.R.: Rostrocaudal gradient of transgene expression in adult skeletal muscle. Proc. Natl. Acad. Sci. USA 88 (1991) 5847–5851.

Grieshammer, U., Sassoon, D. and Rosenthal, N.: A transgene target for positional regulators marks early rostrocaudal specification of myogenic lineages. Cell 69 (1992) 79–93.

Heim, R., Cubitt, A.B. and Tsien, R.Y.: Improved green fluorescence. Nature 373 (1995) 663–664.

Innis, M., Gelfand, D., Sninsky, J. and White, T. (Eds.): PCR Protocols. Academic Press, New York, NY, 1990.

Inouye, S. and Tsuji, F.I.: *Aequorea* green fluorescent protein. Expression of the gene and fluorescence characteristics of the recombinant protein. FEBS Lett. 341 (1994) 277–280.

Lin, S., Gaiano, N., Culp, P., Burns, J.C., Freidmann, T., Yee, J.-K. and Hopkins, N.: Integration and germline transmission of a pseudotyped retroviral vector in zebrafish. Science 265 (1994) 666–669.

MacGregor, G.R. and Caskey, C.T.: Construction of plasmids that express *E. coli* β-galactosidase in mammalian cells. Nucleic Acids Res. 6 (1989) 2365.

Prasher, D.C., Eckenrode, V.K., Ward, W.W., Prendergast, F.G. and Cormier, M.J.: Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 111 (1992) 229–233.

Rosenthal, N., Kornhauser, J.M., Donoghue, M., Rosen, K.M. and Merlie, J.P.: Myosin light chain enhancer activates muscle-specific, developmentally regulated gene expression in transgenic mice. Proc. Acad. Sci. USA 86 (1989) 7780–7784.

Solnica-Krezel, L., Schier, A.F. and Driever, W.: Efficient recovery of ENU-induced mutations from the zebrafish germline. Genetics 136 (1994) 1401–1420.

Wang, S. and Hazelrigg, T.: Implications for bcd mRNA localization from spatial distribution of exu protein in *Drosophila* oogenesis. Nature 369 (1994) 400–403.

Westerfield, M.: The Zebrafish Book. University of Oregon Press, Eugene, OR, 1993.

EXHIBIT 10

Recent Advances in Marine Biotechnology
Volume 4
Pages 77-145
Milton Fingerman, ed.

THE MOLECULAR GENETICS OF TRANSGENIC FISH

Perry B. Hackett[1] and M. Carmen Alvarez[2]

[1]Department of Genetics, Cell Biology and Development
University of Minnesota
1445 Gortner Ave.
St. Paul, MN 55108-1095
USA
Tel: (612) 624-6736
Fax: (612) 625-5754
E-mail: perry@biosci.cbs.umn.edu

[2]Dp de Biologia Celular y Genetica
Facultad de Ciencias
Universidad de Malaga
29071, Malaga
Spain
Tel: 34-95-2131967
Fax: 34-95-2132000
E-mail: Alvarez@uma.es

THE MOLECULAR GENETICS OF TRANSGENIC FISH

P.B. Hackett and M.C. Alvarez

Abstract: The need to develop new stocks of fish for aquaculture is increasing every year. One contribution to meeting future needs is to develop new lines of super fish that will have improved growth rates and increased resistances to environmental stresses and antagonists. Transgenic fish have been made over the past 15 years. The results have taught genetic engineers many lessons and have identified unsuspected problems. Here we review the reasons for making transgenic fish, the methods used to produce such fish, the results from the first and second waves of fish genetic engineering, and the new directions in which genetic engineering in fish is likely to go in the next decade. These new directions and demands for organisms will depend on the employment of new technologies and genetic tools that are just emerging. Development of transgenic fish has an added value, that is, of using fish as a model system for studies of the molecular genetics of growth and development of all vertebrate animals.

Introduction

The most recent review from the United Nations Food and Agriculture Organization concludes that nearly two-thirds of the world's fisheries are either in their peak production stages or in senescence. Moreover, even though some forecast that it may be possible to increase the production of fish from the current plateau of about 90 million tons per year to 110 tons per year; the possibility for increases are based on unreliable data (FAO, 1997). A major part of the problem is that the world's aquatic resources are being exhausted by over harvesting of fish and shellfish. The annual U.S. consumption of about 20 kg of fisheries products per person, which is expanding with increasing appreciation of the dietary benefits of fish, requires the harvest of a total of about 6 million metric tons per year (Parfit, 1995). To meet such demands, which are worldwide, fishing fleets are harvesting more fish and depleting wild fisheries (*e.g.*, Roughgarden and Smith, 1996; Cook et al., 1997; Malakoff, 1997). Establishing moratoria on fishing is economically and politically difficult (Spurgeon, 1997; Masood, 1997a, b; Schmidt, 1997; ScienceScope, 1998), and the strategy will not result in increased harvests in the long run.

The contribution of fish raised in aquacultural facilities is providing an increasing percentage of consumed fish, currently about 20 percent of the total fish harvest. In the U.S., aquaculture of catfish predominates over other types of fish. Catfish feed is about 28 percent protein, consisting of as little as 2 to 4 percent fishmeal. Because the feed conversion rate is as high as 2:1 (Li and Lovell, 1992), fish farming produces more fish than are consumed as food. Carp and tilapia farms comprise the bulk of aquaculture elsewhere in the world. However, it is becoming apparent that aquaculture sometimes is inefficient and it can be ecologically harmful. For instance, between 1986 and 1997, the annual production of farmed salmon, outside of the United States, increased from less than 100,000 metric tons to about 644,000 metric tons valued at about $2 billion. Their value was due in part to the high quality of feed, consisting of about 45% fishmeal and 25% fish oil, which are required at about a 2.8:1 (weight:weight) ratio of input food to harvested fish. Thus, about 1.8 million metric tons of fish were required to produce 644,000 tons of fish for the table (Naylor et al., 1998). Other problems with farmed fish include their high densities of growth, which can lead to environmental pollution and, even in some cases, to abandonment of aquaculture farms (Masood, 1997c).

New solutions are necessary to achieve larger fish harvests, which require lower inputs of fish as food, under controlled environments at high density. Part of the solution must include the fish themselves. Creation of genetically superior stocks of fish that can be farmed at a faster rate for lower cost is needed. There are three methods to achieve improved stocks of fish and other commercially important animals. The first is classical breeding, which has worked well for land animals, but it takes decades to make major changes. Breeding programs for fish require large and expensive facilities. Nevertheless, by controlled breeding, growth rates in coho salmon

(*Oncorhynchus kisutch*) increased 60% over four generations (Herschberger et al., 1990) and body weights of several types of fish have been increased 10 to 30% over three generations (reviewed in Dunham and Devlin, 1999). The second method is marker-assisted selection, a method that utilizes specific genetic markers associated with desirable traits to improve breeding. The method is discussed in the section on the third wave of genetic engineering. The third method is genetic engineering, a selective process by which genes, *whose behaviors we think we understand*, are introduced into the chromosomes to give an organism a new trait or characteristic. The most frequent trait sought so far in fish is improved growth rate (reviewed in Iyengar et al., 1996; Dunham and Devlin, 1999). The results of genetic engineering have exceeded those of breeding in some cases. In a single generation, the following groups report significant increases in body weight have been achieved: 1) 50 to 90 percent in common carp and tilapia with trout or tilapia growth hormone genes, respectively (Chen et al., 1993; de la Fuente et al., 1998). 2) 200-400 percent in tilapia with extra salmon growth hormone genes behind the promoter for the ocean pout anti-freeze protein gene (Rahman and Maclean, 1998b). 3) More than 1000 percent in salmon with the Chinook salmon growth hormone gene and anti-freeze gene promoter (Du et al., 1992b; Devlin et al., 1994a, 1995; Devlin, 1997). The advantage of genetic engineering in fish is that a desired trait can be directly targeted if the appropriate gene has been identified.

The need for superior fish stocks with rapid growth rates and other traits, spawned several efforts to genetically engineer fish. In 1985 fish physiologists and geneticists produced three important "first papers" for genetic engineering in fish: 1) Zhu et al. (1985) reported the first transfer of a single gene into fish that appeared able to transmit the transgene through the germline. 2) Gill et al. (1985) showed that enhanced levels of growth hormone in fish tissues led to increased growth rates. 3) Sekine et al. (1985) reported the first clones of a fish growth hormone gene. It must be noted that the first example of genetic transfer of DNA into fish cells occurred earlier with heterologous fragments of *E. coli* DNA (Vielkind et al., 1971) and large fragments of chromosomal DNA containing a locus that caused formation of abnormal melanophores (tumors) in Xiphorphorine fish (Schwab et al., 1976; Vielkind et al., 1982). Today the number of genetically engineered species of fish exceeds that of all other vertebrates combined (Pinkert and Murray, 1999; this review). With all of this activity, genetic engineering of fish has been reviewed on a regular basis (Maclean et al., 1987; Maclean, 1988; Hew et al., 1989; Ozato et al., 1989; Chen and Powers, 1990; Cloud, 1990; Guise et al., 1991; Fletcher and Davies, 1991; Houdebine and Chourrout, 1991; Powers et al., 1992a; Moav et al., 1992b; McEvoy et al., 1992; Hackett, 1993; Jiang, 1993; Moav, 1994; Maclean and Rahman, 1994; Gong and Hew, 1995; Iyengar et al., 1996; Chen et al., 1996; Winkler and Schartl, 1997; Hackett et al., 1999).

Here we briefly review the past to determine the problems that remain to be overcome in order to obtain the types of fish that will be so urgently needed in the future. Accordingly, we

will include some techniques, under development using laboratory fish that serve as inexpensive models for obtaining information necessary for elucidating genetic programs that determine growth, development, and health. These fish are ideal for developing new methods and technologies for gene transfer and gene inactivation because they 1) can provide copious embryos and offspring on a daily basis, 2) have relatively short generation times, 3) allow non-invasive examination of embryonic development and gene expression in real time on a cell-by-cell basis, and 4) are inexpensive to rear and maintain. In contrast, estimated costs of making a transgenic livestock animal range from about $60,000 to $500,000 with current technologies (Moffat, 1998). Consequently, studies of genetic engineering in fish not only will lead to greater versatility in the genetic re-programming of commercial fish but will also provide insights into the genetics of growth and development in every other vertebrate, including humans.

This review is divided into three principle sections that describe "waves" of activity in the genetic engineering of fish. The three waves are roughly chronological but, as with physical waves, activity in one place may be earlier or later than in another as the wave of ideas and technology flowed between various laboratories. The first wave of activity, lasting about ten years, covered the first attempts at transferring genes that would be expressed in fish and their progeny. The second wave continues through the present, when fish geneticists attempted to increase efficiency and sophistication of transgenesis. The third wave, which is just now beginning, encompasses future studies in the molecular genetics of transgenic fish.

Fish Genetic Engineering: The First Wave

Two sets of studies jump-started the making of transgenic fish. First Gill et al. (1985) showed that growth hormones from non-piscine vertebrates could accelerate growth when injected directly into fish tissues (also see Down et al., 1989; Skyrud et al, 1989). This demonstrated that the proteins encoded in mammalian growth hormone genes could stimulate growth in fish. Second, based on the gene-transfer experiments of Gordon and Ruddle (1981), Palmiter et al. (1982) had shown that transfer of genes into one-cell embryos could lead to enhanced rates of growth, which led to transgenic experiments in farm animals (Hammer et al, 1985). Within months, Zhu et al. (1985) reported the first evidence of growth-enhanced fish and other reports quickly followed. The first wave of transgenic fish studies concentrated on gene transfer and subsequent expression in fish cells. The steps included 1) testing transcriptional regulatory sequences, 2) linking them to various genes, 3) introduction of the recombinant DNA constructs into fish cells and embryos, and 4) examining the expression patterns of the transgenes in tissue culture and fish. In this section we will concentrate on the lessons that were learned from these early experiments.

1. General steps to make and analyze transgenic fish

a. Transgenic DNA constructs

The first step in making a line of transgenic fish is to prepare a specific recombinant DNA construct. The construct contains both a gene encoding the protein of interest and genetic regulatory elements, which direct expression of the gene. Genetic engineering of fish for economic and/or scientific purposes generally requires the ability to express a protein of interest in the desired tissue(s), at the desired time(s) and at an appropriate level. To achieve these objectives the gene must be cloned into a vector that has the appropriate expression capability. Here we describe some of the considerations that were made in constructing recombinant DNAs for transgenic fish.

To obtain sufficient amounts of a transgenic construct, it must be cloned into a vector that replicates to high numbers in a microorganism that can be grown at high density. All of the vectors used in fish genetic engineering are plasmids that can be replicated in *Escherichia coli* to provide more than 10^{12} recombinant plasmids/ml, enough to engineer about one million fish.

In many, but not all, transgenic experiments, the plasmid (also known as the "backbone") was removed from the DNA before transfer to cells. There are two major reasons for this, both resulting from the prokaryotic origin of plasmids. The first is scientific. Removing the bacterial DNA should remove any genetic effects of the prokaryotic DNA on the eukaryotic transcriptional elements. Bacterial DNA often has a high occurrence of CG-dinucleotide base pairs, which in vertebrates can be methylated. Methylation has been correlated with gene inactivation (Riggs, 1975; Jones, 1999). CG's are relatively scarce in eukaryotic genes, except around certain control regions (Tazi and Bird, 1990). Transcriptional inactivation can spread from areas containing methylated cytosines to adjacent regions and thereby reduce expression of neighboring genes (Kass et al., 1993; Hsieh, 1997). One study has shown the relationship of transgene methylation and loss of expression in fish (Gibbs et al., 1994a). The second reason for removing plasmid sequences was politically and philosophically based. There were concerns that fish genomes should not be exposed to prokaryotic DNA since this would be unnatural and hence risky in terms of unknown effects resulting from the intermingling of eukaryotic and prokaryotic DNA. Note, that here are about 10^{13} to 10^{14} bacterial genomes in each human and the same density probably infest fish. Thus, fish are inundated in prokaryotic DNA. Nevertheless, nearly every line of transgenic fish prepared for commercial (but not scientific) purposes has had the plasmid sequences removed from its transgenic construct.

1) Genetic regulatory sequences.

Of the estimated 100,000 genes in vertebrate genomes, about 12,000 are expressed in every cell (Hastie and Bishop, 1976; Adams et al., 1992), most of which are housekeeping genes that are required for simple cellular functions. That leaves about 2,000 genes that direct each cell to perform its specialized functions in a particular tissue. Consequently, in most cells, most genes are off most of the time and are only activated for expression in response to precise signals. Genetic engineering of fish requires an appreciation that the default state of a gene is "off."

Genetic regulatory elements are sites required for initiation of transcription, termination of transcription, RNA splicing and initiation of protein synthesis. The genetic elements regulating initiation of transcription will determine when, where and how much expression will occur. These signals have been the focus of considerable study by many labs. In contrast, mRNA processing steps, such as transcriptional termination and splicing, were thought to be ubiquitous and uncomplicated. Consequently, the splicing and termination signals from a primate virus, SV40 (Huang and Gorman, 1990a), were often used in transgenic constructs delivered to fish. Further studies (Chabot, 1996; Kramer, 1996; Neugebauer and Roth, 1997; von Hipple, 1998) have increased our appreciation of how subtle variations in these elements can be used to regulate gene expression. Consequently, there have been efforts to use piscine elements in "all-fish" transgenic vectors (*e.g.*, Liu et al., 1990d; Du et al., 1992b; Cavari et al., 1993b).

Basal promoters direct only marginal, if any, transcription of downstream genes. To be transcriptionally active, a promoter requires other sites, called enhancers and silencers, which bind regulatory proteins to modulate transcription (Blackwood and Kadonga, 1998; Yuh et al. 1998). The *trans*-acting transcriptional proteins can contort DNA (Muller et al., 1989) and alter or reposition nucleosomes (Lowary and Widom, 1997; Struhl, 1998). Some sequences can act as both enhancers and repressors and a regulatory protein can increase or depress transcription (*e.g.*, Essner et al., 1997, 1999). A key finding was that any enhancer appeared to be able to drive any promoter (Kermekchiev et al., 1991; Pollock and Gilman, 1997). The last concept suggested that regulatory components from genomes of land vertebrates would be active in fish, thereby making genetic engineering of fish possible without a great deal of work in isolating genes and their promoters from fish.

As a result, an initial goal was to determine the relative strengths of various enhancer/promoter complexes, from land vertebrates and their viruses, in fish. In an earlier review (Hackett, 1993), enhancer/promoter strengths were estimated from activities found in tissue-cultured cell lines and in transgenic fish. These estimates were premature. The evaluations depended on comparisons of transcriptional activities from many sources. However, the amount of an mRNA in a cell or fish reflects the difference in its rate of synthesis and its rate of degradation, neither of which is easily quantifiable. Accordingly, enzymatic activities of reporter genes such as chloramphenicol acetyl transferase (*cat*), β-galactosidase (from the *lacZ* gene) and

luciferase (*luc*), which can be measured accurately in small amounts, are often used as a measure of RNA expression. Theoretically this approach is not rigorous because variable processing and degradation rates may independently affect protein accumulation (Huang and Gorman, 1990b). But, there has been a much greater problem in practice -- enzymatic assays are nonlinear with enzyme concentration so that once a substrate is completely converted there is peak activity regardless of enzyme excess. In most of the publications describing relative promoter activities in fish, the displayed CAT conversion levels were in excess of 60 percent, well within the non-linear range of the assay (Moav et al., 1993), and almost never were standard activity curves employed to correct the raw data.

Table 1 shows a compilation of the putative transcriptional strengths of various enhancer/promoter combinations using reporter genes in tissue culture. The most common basal promoter was that from the herpes simplex virus thymidine kinase (tk) gene, which was often linked to the CMV promoter or to inducible heat-shock and metallothionine promoters (Table 1). The ratings of enhancer-promoters in Table 1 must be viewed with caution even though many labs have examined them in many cell lines. One sees that there is little agreement between labs on the efficacy of using either of three well-studied viral promoters in fish. In the comparisons, we arbitrarily used the transcriptional regulatory sequences in the long terminal repeat sequence of the avian Rous sarcoma virus (RSV) as a standard. It can be seen from the reports of viral promoters in Table 1 that the enhancer/promoter complexes from the primate cytomegalovirus (CMV) and SV40 virus, as well as β-actin genes from several sources, have reported activities that vary as much as 100-fold in various cell lines and labs. This is quite surprising. These promoters give robust expression and the delivery of the transgenic constructs to the cell lines is relatively easy.

The conclusion we draw is that the RSV, CMV and SV40 promoters are all rather strong, and most importantly, are active in most, but not all, tissues or cells. Likewise, the data in Table 1 demonstrate that several inducible promoters from non-piscine origin are active in a wide variety of fish cell lines, but not always at the same levels. Together these data show that transcriptional proteins in fish and other vertebrates are fairly well conserved, as was suggested early from an examination of the enhancers and promoters of the carp β-actin gene in fish, birds, and mammals (Liu et al., 1990a, b). Despite some suggestions that fish enhancers and promoters are "special" there is no rigorous proof that they work better to direct transcription in fish than the same elements from animal genomes.

Of course the real question is how well these promoters work in fish embryos and their differentiated tissues. The problem seen in tissue culture was exacerbated. Expression from the same viral genetic constructs in fish embryos varied by orders of magnitude in different fish within the same studies, making it impossible to calculate promoter strengths. A compilation of

constructs using reporter genes is shown in Table II. When expression levels in individual fish embryos are provided (*e.g.*, Winkler, 1991, 1992; Sekkali et al., 1994; Alam et al., 1996) variations of 100 to 10,000-fold are seen for the same promoters driving different reporter constructs. This is due to two effects. First, the *lacZ* and *cat* genes have a useful range of about 100-fold, while expression of luciferase can be quantified over a range of more than 10^5. Second, depending on the fate of the transgenes in a given embryo, a wide variation in expression can be obtained. Thus, in Table II, the best we can do is display the wide use of some promoter-gene constructs and whether expression was achieved in the fish into which the constructs were delivered (F_0 expression) and stable (F_1 expression). The presumption is that the approximate levels of expression, achieved from enhancer/promoter complexes in cultured cells, reflect the levels that might be achievable in a whole fish. But, there is no proof of this. What is needed is careful quantitative assessment of expression in fish of the same gene in different tissues and from developmental periods. We will come back to this subject later in discussions of methods for assuring reproducible expression of transgenes.

2) *Genes*

Just about every early transgenic fish study employed a modified gene, often a cDNA that encoded a desirable protein product, linked to a heterologous promoter as described above. The most common genes (cDNAs), besides reporter genes tabulated in Table 2, were those that encode growth hormones (GH). Table 3 lists many of the transgenic constructs of genes and heterologous promoters that conferred "gain-of-function" to transgenic fish. As with the data in Table 2, reliable estimates for the levels of gene expression are nearly impossible to assess, except in rare cases, because of the enormous variation in expression per animal. Hence, one can at best look for expression of the gene product by RNA or protein assays in the F_0 and F_1 generations and for phenotypic effects. The review by Iyengar et al. (1996) does an excellent job in discussing these reporter genes as well as growth hormone and related genes.

The information in a transgene may be delivered as a complete gene, a cDNA, a minigene (a gene that has had one or more but not all of its introns removed), or a mix of a cDNA with sequences from another gene. Many genes are not expressed at their normal levels unless they contain an intron (Brinster et al., 1988; Huang and Gorman, 1990a). Consequently, the earliest expression vectors made for mice included an intron that could function for intact genes, mini-genes and cDNAs (Hammer et al, 1985). The reasons for needing introns are still not clear. Several introns, such as the first intron in the β-actin genes of vertebrates, contain enhancer elements that can facilitate transcription (Liu et al., 1990a,b,c) as well as RNA stability. Bétancourt et al. (1993) examined the efficiencies of gene expression in some fish cell lines to determine if "fish introns" were more useful than those from the genomes of land vertebrates. They found that the SV40 VP1 intron inhibited expression in some lines whereas the small-t

intron from the early SV40 transcript did not. But, given the wide range of findings from cell lines (Table 1), the only reliable conclusion that could be drawn was that specific introns might work better or worse in different cell types. These findings agree with current thoughts of intron removal as a function of tissue type (Leff et al., 1986). Certain introns, like those of the β-actin genes, appear to be well conserved and spliced in many cell types (Liu et al., 1990a). Introns generally are more heterogeneous in sequence than exons and so are a good source of genetic polymorphisms in fish (Chow, 1998). As with introns, after initial use of SV40 and other transcriptional termination and polyadenylation signals from genes of land animals, fish geneticists have turned to using fish genes as sources for these regulatory motifs (Liu et al., 1990d; Du et al., 1992b; Cavari et al., 1993b).

b. Methods of Transgene Delivery

After assembling the components of the transgenic construct, it must be delivered to the nuclei of cells in the developing embryo in order for it to be expressed and distributed to all tissues of the fish. A screening process for those fish with active transgenes had to be established because not all deliveries of the transgene are effective and not all constructs behave in the desired manner. Procedures for these two steps were adapted from technologies used in land animals. Here we describe those procedures and some of the necessary benchmarks that were required for determining whether transgenes were effectively delivered to chromosomes of living fish embryos.

1) Microinjection.

As noted earlier, the first wave of transgenic fish followed procedures used to make transgenic mice. However, methods that are reasonable for mouse eggs are not necessarily ideal for fish embryos. Mouse oocytes require a substantial amount of work to orient, inject, and implant *in utero*. In contrast, fish eggs are abundant, large and have pronuclei that are nearly impossible to find, surrounded by hard chorions, undergo more rapid early cell-division, and often develop outside of their mothers. Nevertheless, because of its reliability, microinjection is the most popular form of gene transfer into fish embryos (Tables 2 and 3). Microinjection techniques were developed to target delivery of transgenic DNA to the pronuclei of amphibian and mammalian embryos. Transgenes integrate into chromosomes in about 25 percent of mouse embryos but less often in the genomes of other animals (DePamphilis, 1988). Microinjection is relatively efficient with mouse eggs since the cells are small, the pronuclei are visible, and the chorions of the eggs are soft and easily penetrated by the microinjection needle. In contrast, fish eggs are about 1000-30,000 times larger than mammalian eggs and the pronuclei make up about 0.001 percent of the 1-cell embryo compared with about five percent in a mammalian embryo. The pronuclei can be visualized when fluorescent dyes are injected into the eggs, but ultraviolet light irradiation is

required to locate the exceedingly small pronuclei residing in the vicinity of the very much larger yolk (*e.g.*, Yamaha et al., 1988a). As result, pronuclear injections are often impractical, although in some species such as medaka this technique can be used. To make matters worse, the rapid onset of cleavage and the hardening of chorion following fertilization interfere with visualization of the pronuclei as well as penetration by the injection needles. Injection of DNA into oocytes has been examined (Inoue et al., 1992b; Zhang et al., 1993) but the technique has gained little interest.

In order to allow more injections per needle, and to support alternative delivery mechanisms such as lipofection and electroporation, in some labs the chorion was removed prior to gene delivery *(e.g.*, Zhu et al., 1985; Hallerman et al., 1988; Stuart et al., 1988, 1990; Fletcher and Davies, 1991; Culp et al., 1991; Müller et al., 1992, 1993). However, the procedure is labor-intensive and slow, which reduces the number of embryos that can be injected. Success has been mixed. On the whole, embryo survival and transgene integration rates do not appear to be much better than those obtained by simple microinjection without chorion removal. In some cases using salmon and tilapia eggs, microinjection has been through the micropyle (e.g., Shears et al., 1991; Indig and Moav, 1992; Rahman and Maclean, 1992a). For hard chorions, success has been achieved by drilling a small hole in the chorion (Rokkones et al. 1985).

The difficulty of localizing pronuclei led to injection of transgenes into either the embryonic cytoplasm or even into the yolks, in the latter case depending on a streaming phenomenon to deliver the DNA to the cytoplasm. Working with zebrafish embryos, Williams et al. (1996) have noted the high activity of expression of foreign DNA in the yolk syncytial layer, which contains giant polyploid cells that appear to regulate embryonic cell movements but are not part of the embryo *per se*. In most of the studies reported in Tables 2 and 3 between 1985 and 1995, about 10^6 to 10^7 molecules of DNA in an aqueous, buffered volume of 1 to 2 nl (Fletcher and Davies, 1991; Hackett, 1993) were injected into embryos or fertilized eggs. This is a relatively large volume of solution, about 20 to 100 times the average nuclear volume of fish nuclei; but, so much free water appears to have little effect on survival (Hackett, 1993). The large 1-cell embryo may accommodate this abnormal influx of aqueous DNA by forming an "artificial nucleus" as has been seen in *Xenopus* embryos (Newport, 1987). Indeed, microinjected ethidium bromide-stained DNA appears to remain as a distinct aggregate in zebrafish embryos (Westerfield et al., 1992). A detailed description for microinjection of zebrafish embryos that yields state-of-the-art success can be found in Meng et al. (1999). Thus, in most instances microinjection is used to overcome the first barrier shown in Figure 1. The hope was that some of the injected transgenic DNA would make its way into chromosomes.

Direct injection of transgenic DNA into adult fish tissues, generally muscle, is another form of injection that will be discussed later in the section on Genetic Vaccines. In this case, the

transgenic DNA will make its way into a group of cells in the neighborhood of the site of injection where it may be expressed locally. An undefined number of cells will be transformed, which may be sufficient to establish immunity. In contrast to the microinjection into very early embryonic cells, the transgenes have virtually no chance of being passed on through the germ-lines into progeny. Experiments in which DNA was microinjected into muscle tissue are designated (MI) in Tables 2 and 3.

There is an enormous variation in the various reports on success of microinjection. Our analysis of the results from the many experiments, which employed a wide variety of genes, promoters, and methods of assay, shows the following: 1) In the better studies, embryonic survival often decreased less than about 10 to 20 percent when less than about 100 pg (ca. 10^7 copies of DNA) were injected. 2) About 50 percent of the eggs were able to express the transgenic DNA shortly after gene transfer but expression rapidly decreased after a few days. 3) Transgenic DNA integrated into the genomes of relatively few fish that developed from microinjected embryos, about 1 percent for large fish and up to about 20 percent for zebrafish and medaka. 4) All of the transgenic fish were mosaic for the presence and/or expression of the transgene, indicating that integration of the injected DNA occurred late in embryonic development. Trained investigators can inject fish eggs at rates up to several hundred per hour, depending on the species, the logistical support, the accuracy of injection and the level of training and experience. Thus, the large numbers of fish embryos that can be injected offsets the low rates of transgenesis per fish. However, spawning in commercially important fish is restricted to a specific period, and the zygotes begin rapid cell division within a few hours of fertilization. As a result of the labor required for microinjection and subsequent screening for those few fish with expressible transgenes, four other methods for delivering genes to fish embryos were tried in the first wave of transgenesis in fish: electroporation, DNA-binding to sperm, ballistics, and lipofection. The results from microinjection are standards against which the other gene-transfer techniques are compared in Tables II and III.

2) Electroporation.

Electroporation is a standard method for gene transfer to cells in tissue culture (Neumann et al., 1982; Shigekawa and Dower, 1988). It has been tried in several species of fish (Xie et al., 1989; Inoue et al., 1990; Inoue, 1992; Buono and Linzer, 1992; Müller et al., 1993; Lu et al., 1992; Powers et al., 1992b; Zhao et al., 1993; Murakami et al., 1994; Ono et al., 1997; Zhang et al., 1998). In all of the studies except that by Zhang et al. (1998), the chorions were removed, a time-consuming process that defeats the objective of mass transfer of DNA without treatment of each embryo. Although embryo survival is lower than with microinjection, transgenic DNA can be transferred and expressed in up to 60 percent of the fish that survive larval development. Nevertheless, there are problems with procedure. These include wide variations in results, which

could be due to the use of electroporation conditions, exponential decay compared with square-wave electroporators, the developmental stage of the embryos, and poor assays for DNA integration into genomes. The goals of the studies were not always the same, some labs sought high rates of survival whereas others wanted high rates of transformation. Another problem that may have occurred in some studies was incorporation of proteins and other materials in the environment into the eggs (Yamaha et al., 1988b). Looking at the reports as a whole (Tables 2 and 3), electroporation has not caught on with most labs.

3) Sperm.

A more natural method for mass transfer of DNA to embryos was tried in mice. That was to coat sperm with transgenic DNA so that the lucky sperm would transfer the exogenous DNA during fertilization without any ill effects on the embryo (Lavitrano et al., 1989). Several fish labs tried sperm-mediated transgenesis before Brinster et al. (1989) reported their inability to reproduce the results. DNA does bind to sperm (Arezzo, 1989; Atkinson et al., 1991; Castro et al., 1991) and can be taken up into fish embryos (Khoo et al., 1992; Chourrout and Perrot, 1992; Patil and Khoo, 1996), but the genes are either not expressed or only soon silenced. The fate of the DNA-bound to sperm is important. There is likely to be considerable free DNA from many sources (broken cells, microorganisms, etc.) which could bind to sperm during normal mating. Such foreign DNA would be hazardous to the recipient embryo if it had a chance to integrate into the embryo's genome. However, Patil and Khoo (1995) have shown using *in situ* hybridization that some of transgenic DNA is internalized into the sperm head, although its state is unknown. In any case, normal protection mechanisms against invading DNA sticking to sperm coats is likely to keep this form of delivery from being practical. Very recently a new method has been tried, intra-cytoplasmic injection of detergent-disrupted sperm and sperm-heads (Perry et al., 1999). Up to twenty percent of transgenic mice produced offspring that expressed transgenes. The sperm heads were "considered dead due to disrupted membranes" but still supported full development. This method still requires microinjection and thus can not be considered a mass transfer procedure.

An attempt to combine the theoretical advantages of two of the above techniques has been tried -- electroporating sperm prior to fertilization (Müller et al., 1992; Sin 1993, 1998; Tsai et al., 1995b). The nature of the transgenic DNA following electroporation with fish sperm results in greater uptake of transgenic DNA into the sperm heads, but most still remains on the exterior surface as evidenced by its susceptibility to DNases (Symonds et al., 1994; Patil and Khoo, 1996). Most studies using electroporated sperm have not looked at expression or germ-line integration. At this point, the difficulties in the procedure appear to be greater than the benefits. To circumvent the problem with protection of the transgenic DNA from natural defense mechanisms in the egg, the transgenic DNA could be encapsulated in a viral coat and then bound

to sperm; this works with natural viruses such as infectious hematopoietic necrosis virus (Mulcahy and Pascho, 1984).

4) Biolistics.

When subtle means fail, some resort to force. This is the case with gene transfer into plants where there is a tough cell wall surrounding each cell (Klein et al., 1987). Bombardment of cells with DNA coated particles, biological ballistics, was tried with fertilized loach, zebrafish and rainbow trout eggs, resulting in high mortality and low expression rates (Zelenin et al., 1991). However, their assay for success in some experiments was survival of the embryo in a G418 solution. But, because almost all transgenic founder fish mosaically express transgenes, it is likely that some tissues would be susceptible to the G418, leading to death of the embryo (Yoon et al., 1990). Thus, their assay for success was the most demanding of all so far employed by fish geneticists. Ballistic transfer of DNA has also been used in sea urchins (Akasaka et al., 1995) and has been applied to direct introduction of DNA into muscle cells of adult fish (Gómez-Chiarri et al., 1996a). The latter technique may be useful for genetic immunization against disease-causing microorganisms. Besides injury to the cell, a major problem with the ballistic procedure may be difficulties of the foreign DNA leaving the delivery particle once in the cell. As an alternative, electrospray delivery has been attempted in zebrafish. For this, a fine mist of water containing transgenic DNA was electrostatically propelled onto a plate containing hundreds of embryos. Expression of the transgenic constructs was never detected, even at voltages that killed the embryos (P. Hackett and S. Ekker, unpub.).

5) Lipofection.

Nucleic acids encapsulated in synthetic lipid vesicles can be taken up into cells and tissues of animals where they can be expressed (*e.g.*, Bandyopadhyay et al., 1998; Kren et al., 1998). In fish, several cocktails of DNA and proteins have been mixed and used to deliver transgenic constructs to dechorionated zygotes at the 2-16 cell stage (Szelei and Duda, 1989; Szelei et al., 1994). As with the sperm methods, the DNA could get into the embryos, but it was soon lost and its expression was transitory.

Together, the results of all of the transgenic procedures emphasize an important point, that is, delivery of transgenic DNA is only one half of the problem, the second half is to achieve rapid integration that leads to stable dispersal of the construct in an expressible state to all cells. The second half of the problem requires a biological activity that will overcome the second and third barriers shown in Figure 1 by catalyzing the integration of transgenic DNA into chromosomes. Methods that attacked this problem were a primary concern in the second wave of genetic engineering in fish.

c. Expression, Integration and Inheritance Assays

With microinjection the amounts of DNA delivered to the average embryo can be better quantified than with the alternative procedures. In most studies, about 1 to 10 million molecules are delivered to each embryo. Yet at best only a very few genomes take up even a single copy of DNA, which is an efficiency of incorporation into chromosomes of less than 10^{-4} percent. The low efficiency is due to protective mechanisms that cells have to keep foreign DNA from recombining with genomes. One of these mechanisms in some fish is the rapid onset and blinding speed of DNA replication in early development. For instance, the period of DNA replication (S-phase) in human cells requires a minimum of at least 5 hours in tissue culture at 37°C and is slower during embryonic development. In contrast, the zebrafish genome, whose size is about 60 percent that of a human, replicates every 15 minutes at 30°C for cleavages 3 through 10. There is probably no G1 or G2 phase in these early cell cycles. To accomplish such rates of replication, there must be about 100 times as many DNA polymerases per unit length of fish chromatin as mammalian chromatin. Transgenic DNA also is replicated (Stuart et al., 1988), indicating that DNA replication does not depend on specific DNA sequences (ori sequences) that are required for DNA synthesis later on (Hyrien et al., 1995). It is not until after midblastula transition that DNA replication slows and transcription of zygotic genomes begins in *Xenopus* (Newport and Kirschner, 1982a, b), zebrafish (Kane and Kimmel, 1993), medaka (Tsai et al., 1995a) and seabream (García-Pozo et al., 1998). The onset of transcription appears to mark a time when chromatin replication slows, the DNA is not so packed with DNA polymerases and thereby becomes available for enzymes that mediate integration. Fighting for access to chromatin never stops in nuclei (Prioleau et al., 1994).

There are two important consequences of so few transgenic DNAs making their way into chromosomes. First, the integrating copies of transgenic DNA are diluted more than a million-fold with unintegrated DNA of identical sequence, which makes analysis of the integrated DNA exceedingly difficult. The unintegrated DNA can recombine, at variable rates that depend on the input conformation of the transgenic DNA, to form a variety of concatemers (reviewed in Hackett, 1993; Iyengar et al., 1996). Second, virtually all of the truly transgenic F_0 fish were mosaic (Westerfield et al., 1992; Hackett, 1993). As a result, selecting transgenic fish is quite labor intensive, requiring the raising of all embryos subjected to transgenic DNA until gametes can be accurately assayed for the presence of integrated foreign DNA.

In the early years of fish transgenesis, a variety of assays were used to determine whether integration had occurred. These included the following: 1) demonstration of transgenic DNA in embryos and adults by dot-blotting or PCR amplification, the assumption being that transgenic DNA would be unstable and/or diluted during cell division; 2) expression of the transgene after larval development, the assumption being the same as before; 3) Southern blotting to show that the size of the transgenic DNA changed as a result of its integration into chromosomes; and 4)

detection of transgenic DNA or its expression in F_1 progeny. None of these assays was sufficient. Owing to the enormous amounts of unintegrated DNA, many molecules of which recombined in unknown ways, transgenic DNA size is insufficient to demonstrate integration when detected by blotting or PCR amplification. Some integrated transgenes were not expressed due to their integration into heterochromatin or into regions under the control of silencers of their enhancers and, conversely, some constructs can be expressed from unintegrated copies (Hackett et al., 1999). Stuart et al. (1988, 1990) showed that unintegrated transgenic DNA was passed as episomes into F_1 progeny. The problems associated with the persistent presence of unintegrated transgenic DNA in fish have been reviewed (Hackett, 1993; Iyengar et al., 1996).

There are three methods that are reliable indicators of integration of transgenes into fish chromosomes. The first is chromosome in situ hybridization in which tagged probes are hybridized to metaphase spreads of fish chromosomes (*e.g.*, Tewari et al., 1992). The second method uses any of several forms of linker-mediated PCR or inverse PCR (*e.g.*, Ivics et al., 1993, 1997) to determine the sequence of the locus into which the transgenes integrated. The third method is less direct, to establish Mendelian segregation of the transgenes in progeny. Even better is to show that expression is passed in a Mendelian fashion in progeny. Relatively few studies have done this (Tables II and III). Overall, in the early days there were many claims of integration of transgenes, but little rigorous proof.

2. Accomplishments of the First Wave

a. Basic Science

At this point, the molecular genetics of transgenesis in fish might look a bit bleak as a result of the emphasis on the inefficiencies of gene transfer and expression. However, the results of so many studies, especially those listed in Table 3, provided a wealth of scientific information and techniques that were used to study the developmental genetics of zebrafish and medaka. Initially, transgenesis of commercially important fish looked at two characteristics for improvement - growth enhancement and cold tolerance. These areas are evaluated here.

b. Growth Enhancement

Three aspects of enhanced growth in fish were examined for economic purposes: 1) increased growth leading to earlier maturation, 2) enhanced growth size per adult fish, and 3) improved feed efficiency. At first, the transgenic constructs containing human, bovine, or rat growth hormone genes linked to either viral or mammalian metallothionein (heavy metal-inducible) promoters. Growth enhancements were modest, but acceptable, in the range of 10 to above 50 percent in carp, trout, salmon, and northern pike (Agellon et al., 1988; Chen et al., 1993; Cui et al., 1993; de la Fuente et al., 1995; Gross et al., 1992; Lu et al., 1992; Hernández et al., 1997; Martínez et al., 1996; Zhang et al., 1990; Zhu, 1992;). However, the viral and heavy

metal-inducible promoters were unacceptable because of their alien status in fish cells and their associations with disease. Generally the results were averaged over populations of fish, without regard to parameters that could affect size, including sex, rearing conditions, expression levels of the growth hormones, birth orders, etc. For instance, Gross et al. (1992) examined transgenic northern pike containing an RSV-bGH construct. They found sex-linked differences in weight gains compared with controls. A second potential problem was variations in rearing conditions between labs using the same or different transgenic DNAs in different species of fish. In many cases fish were raised indoors under artificial conditions that may not have been optimal for enhanced fish growth.

The situation changed dramatically at the end of the first wave. New vectors with regulatory elements from fish were employed (Liu et al., 1990d; Du et al., 1992b; Cavari et al., 1993b). The promoters ranged from those from the ocean pout and winter flounder antifreeze protein genes (weak promoters) to the modified carp β-actin promoter (strong). The first lines of transgenic salmon had an opAFP promoter directing the Chinook salmon growth hormone gene, which resulted in 100 to 500 percent weight increases in the fish (Du et al., 1992a; Davies et al., 1989). These results were followed by spectacular growth enhancements of over 1000 percent in Pacific salmonids using the same types of constructs (Devlin et al., 1994, 1995a,b). There appears to be increased feed efficiency in the growth-enhanced transgenic carp containing human growth hormone (Fu et al., 1998).

The above results marked a successful outcome to the first wave of transgenesis in fish – a primary goal had been reached by a couple of labs in a reproducible manner. The explanation for these results is not entirely clear. It appears that the strongest promoters may not be the best for achieving particular phenotypes. Comparisons of growth enhancement with levels of transgenic growth hormone showed an inverse correlation (*e.g.*, Du et al., 1992a; Hernandez et al., 1997; de la Fuente et al., 1998). The probable reason for this result is that excess growth hormone saturates all of the growth hormone receptors thereby preventing their dimerization and subsequent activation of signal transduction (de Vos et al., 1992). The message: if you want to fool mother-nature you have to be subtle. The interactions of growth hormones and its relatives are quite complex (Rand-Weaver and Kawauchi, 1993; Heyner and Garside, 1994) and hypothalmic controls must be bypassed (Lira et al., 1988). One side benefit of the transgenic studies will be a greater understanding of the physiology of growth in fish (and presumably other vertebrates) as various parameters of body composition are determined in the various transgenic lines of fish (Chatakondi et al., 1995; Cui et al., 1996). However, one expectation that there may be significant phylogenetic specificity to the activities of growth hormone are not supported by the studies of Cui and Zhu (1993). They found that a expression of transgenic human growth hormone could compensate for the loss of carp growth hormone in hypophysectomized fish.

These data suggest that the earliest trials using heterologous growth hormone genes were fundamentally sound; it may have been the strong promoters that limited their successful applications to aquaculture. Dunham and Devlin (1999) have reviewed several factors that may limit growth enhancement in some fish, including the history of the lines and previous efforts to maximize growth within the genetic potential of the fish.

c. Cold tolerance

Much of Canada's marine environment is too cold to permit aquaculture of salmon and other fish. Here, cold-tolerant fish could be economically beneficial. This was the reason for the isolation of the antifreeze protein (AFP) genes (Hew et al., 1988), whose protein products are able to protect fish to about -1.8°C (Davies and Fletcher, 1988; Wu et al., 1998). AFP genes from winter flounder (wf), ocean pout (op), and sea raven (sr) were isolated and their promoters were successfully employed in the growth hormone constructs discussed earlier. The first studies were conducted on the promoters linked to reporter genes (Table II) to determine their strengths and responsiveness to cold conditions (Gong et al., 1991; Du et al., 1992a). Thereafter, Atlantic salmon with transgenic AFP genes were produced that expressed wfAFP constructs (Fletcher et al., 1988, 1992; Shears et al., 1991; Hew et al., 1992), but not to a sufficient level to provide measurably increased survival in cold water. However, in some F_2 lines there was seasonal variation in the expression of the AFP genes (Poon et al., 1999). In contrast to salmon, goldfish harboring the wfAFP construct did show significant resistance to cold such that 67 and 33 percent could survive 6 and 12 hours at 0°C respectively compared to 20 and 0 percent of the control fish for the same conditions. Thus, the problem appears close to resolution. The results do raise an interesting question – why do the ocean pout and winter flounder genomes have so many (around 50) AFP genes with very weak promoters rather than one or a few genes with more powerful promoters. The answer to this question may help explain cold resistance as well as provide important insight into the mechanisms nature employs for physiological defenses against environmental antagonists.

3. Lessons Learned from the First Wave

The results from the first wave indicated that transgenic fish could be made on a relatively large scale, which surpasses that of all other vertebrates together in terms of variety of species and applications. Several major goals, which involved the introduction of single genes under relatively simple enhancer/promoter elements, were achieved. However, it became clear that the rates of making true transgenic F_0 fish were lower than initial claims. Moreover, the F_0 fish almost always had mosaic germ-lines from which F_1 and later generations of fish could be obtained according to Mendelian laws of inheritance. It also became clear that reliable and continued expression of genes could not be assumed, as shown in Tables II and III. As noted earlier, identical constructs could be expressed levels that varied 100- to 10,000-fold (*e.g.*,

Winkler, 1991, 1992; Sekkali et al., 1994; Alam et al., 1996). In some cases, transgenes expressed in F_0 and F_1 fish failed to express in later generations (Caldovic and Hackett, 1995, 1999). A major goal during the second wave of fish transgenesis was to develop procedures that would direct early genomic integration of exogenous DNA at the one-cell stage. A second goal was to find methods for getting the genes to express reliably once they were integrated into chromosomes.

C. Fish Genetic Engineering: The Second Wave

Integration of transgenic DNA into a chromosome, after surmounting the three barriers shown in Figure 1, is not sufficient to guarantee expression of a transgene. Some regions (euchromatin) of chromosomes contain genes whose expression is much higher than in other regions (heterochromatin) where the DNA is rarely if ever transcribed. The determinants of active and inactive domains are not known, but if foreign DNA integrates into heterochromatin, it will not be expressed. This is called "position effect" (Geyer, 1997) and it implies that a DNA sequence that integrates into a site in a chromosome is subject to regulation by elements that normally control gene expression in that region (Dobie et al., 1997). In contrast, when large chromosomal fragments are transferred to rainbow trout (Disney et al., 1988), the genes remain active because their normal chromosomal context is maintained. Furthermore, DNA inserted into a chromosomal site may be active for awhile, but later repressed permanently as a result of methylation. In fish, silencing of transgenes in F_1 and subsequent generations has been noted many times (Hackett, 1993; Caldovic et al., 1999). Thus, in addition to overcoming the three barriers to integration of transgenic DNA at higher rates, reducing position effects and preventing silencing were major goals of the second wave of fish transgenesis.

1. New methods for DNA transfer into chromosomes

Three methods are under development to improve the efficiencies for gene delivery into fish chromosomes. They are the use of nuclear-localizing proteins to help import DNA into nuclei, viruses to assist in overcoming all three barriers, and mobile DNA elements to increase the efficiencies of nuclear uptake and integration of transgenic DNA.

a. Nuclear-localization Peptides

Many viruses use proteins that contain nuclear-localizing sequences (NLSs) mediate the transfer of their genomes into nuclei for transcription. Accordingly, Collas and Alestrom attempted to use a highly basic stretch of amino acids, PKKKRKV, from the large-T antigen protein of primate virus SV40 as a means of facilitating delivery of transgenic constructs into zebrafish nuclei. They were quite successful. At a ratio of about 1000 molecules of the NLS peptide per DNA construct (a luciferase reporter gene behind a CMV promoter), they were able to achieve peak transfer of

the DNA into nuclei of injected embryos. The uptake into chromosomes of NLS-peptide-coated DNA was about 100-fold higher than that of naked DNA (Collas and Aleström, 1997a,b; Collas et al., 1997). Expression of luciferase was equally enhanced such that injection of only 10^4 NLS-peptide-coated DNAs produced enzyme activities that were equal to those from injections of 10^6 naked DNA molecules (Collas et al., 1996). The stability of luciferase expression following passage through the germ line has not yet been reported. This strategy overcame the nuclear membrane barrier, but the real barrier still remained, that was efficient integration into recipient genomes.

b. Retroviral Transduction

Viruses have developed methods for circumventing the three barriers of the cellular and nuclear membranes and chromosomal integrity. However, most viruses are very picky about the cells they infect; they have species and tissue-type specificity that reside in glycoproteins on their viral coats or membranes. There are only a few known fish retroviruses that even have the potential to be used as vectors for gene delivery (Martineau et al., 1992; Holzschu et al., 1995; Hart et al., 1996). However, by replacing the normal species/tissue-specific *env* protein gene of the murine leukemia retrovirus with the G-glycoprotein gene of the vesicular stomatitis virus, a pseudotyped retrovirus has been produced that is able to infect *any* cell whose membrane is available (Burns et al., 1993). The retrovirus is capable of penetrating the cell membrane and it encodes an integrase protein that will catalyze the integration into the genome of the DNA copy of the retroviral genome (provirus). However, the pseudotyped virus cannot infect fish embryos through their chorions so it must be injected. In zebrafish about $10\text{-}20 \times 10^4$ viral particles were injected into blastula-stage embryos (512-2000 cells), leading to several insertions into zebrafish genomes (Lin et al., 1994a). As a result of the injection during blastula stage, all of the embryos obtained were highly mosaic with from 5 to 22 proviral insertions transmitted to F_1 fish. For screening of mutations that affect growth and development, this is a useful strategy because it allows a number of mutations to be screened simultaneously per fish (Gaiano et al., 1996a,b). Accordingly, this method has been used for insertional mutagenesis and identification of several genes (Allende et al., 1996).

A major drawback of using pseudotype-retroviruses as vectors for gene delivery is that the high-titer retroviruses that are required for this procedure do not express transgenes; they have only been effective as insertional mutagens for tagging genes. Moreover, there are questions about efficacy and safety in using retroviruses as vectors in substances that can contact humans (*e.g.*, Smith et al., 1996). These types of viruses are especially tricky because they can infect any cell, including those of the human conducting the experiments.

Because the retroviruses require manual delivery into the embryo by microinjection, an alternative procedure is to just employ the most important ingredient of the retrovirus, its integrase. In this procedure, the transgenic constructs were flanked with DNA sequences that could be recognized by a retroviral integrase. Integrase (or its mRNA that is translated upon entry into the embryonic cells) was added to the transgenic DNA and the mixture injected into 1-cell embryos (Ivics et al., 1993). This procedure increased levels of expression of transgenes from 10- to 40-fold. However, as the experiments were being conducted, it became clear that most retroviral integrase proteins require host factors that limit their activities in alien cell types. Integrase activity also could have been limited because of a need for "activation" of the chromatin by some endonuclease. Nevertheless, the work provided the first insight that recombinases could be employed to enhance integration of transgenic DNA into fish chromatin.

c. Transposon-mediated Transgenesis

Retroviral-mediated integration also has the potential of suffering from some public perception that use of cancer or leukemia causing viruses for gene delivery might be dangerous to consumers. Accordingly, a search was undertaken to find alternative recombinases that might not have the problems associated with retroviral integrases. Obvious candidate enzymes were the transposases that were known to be responsible for the widespread distribution of repetitive DNA-type transposable elements in fish and other animal genomes (Izsvák et al., 1997; Ivics et al., 1999). The class of Tc1/*mariner*-type transposable elements was closely examined because they appeared to be ubiquitous in animal genomes, suggesting that they require few, if any, species-specific host factors. Moreover, these DNA elements move in a simple, cut-and-paste, manner in which a precise DNA segment is excised from one DNA molecule and moved to another site in the same or different DNA (Plasterk, 1993). The excised DNA is flanked by inverted terminal repeats to which the appropriate transposase molecule binds. Both the Tc1 transposon from the nematode *Caenorhabditis elegans* (Vos et al., 1996) and the *mariner* transposon *Mos* from *Drosophila* (Lampe et al., 1996) can transpose cell-free systems in the presence of their respective transposase enzymes made in *E. coli*. This meant that transposons carrying a gene-of-interest could be mobilized by transposase provided in *trans*. In contrast to other DNA transposons (*e.g.*, *P*-elements) that have requirements for specific host cofactors, which limits their usefulness for transgenesis in fish (Gibbs et al., 1994a; Izsvák and Ivics, unpublished), the Tc1/*mariner*-type transposons appeared to be suitable for many genetic applications. However, despite its reported need for just transposase without other cofactors, Ivics and Izsvak were unsuccessful in using the nematode Tc1 element for gene transfer in fish (unpublished). This induced a search for Tc1/*mariner*-type transposons in vertebrate organisms, especially fish.

Fortunately, at about this time, the Emmons lab reported evidence of transposable elements belonging to the Tc1/*mariner* family in salmonids and zebrafish (Radice et al., 1994). However, all of the elements were defective, with gaps, stop codons, and frame-shift mutations in the putative transposase coding sequences. The report led Izsvák et al. (1995) to search for, and find, Tc1/*mariner* elements in a dozen species of fish. Alas, all of the sequences isolated had many mutations and gaps in their transposase genes that inactivated the transposons (Izsvák et al., 1995; Ivics et al., 1996). Nevertheless, the majority of the DNA-type transposable elements in fish could be classified into two major subfamilies, zebrafish- and salmonid-type elements. The two are characteristically different in their encoded transposases and their flanking sequences even though they are clearly derived from a common ancestor that existed millions of years ago (Ivics et al., 1996).

The failure to find an active transposase gene in the search of so many fish elements was frustrating at the time. However the searches yielded a sufficiently large number of sequences so that a theoretical sequence could be derived from phylogenetic principles. Accordingly, a salmonid sequence that had about 96 percent identity to the consensus salmonid sequence was used as a starting point for a series of site-specific mutagenesis steps that culminated in the synthesis of the *Sleeping Beauty* (SB) transposon system (Ivics et al., 1997). The SB transposon system was shown to be able to enhance integration from 20 to 40-fold in mammalian cells (Ivics et al., 1997) and about 20-fold in zebrafish embryos (Izsvák et al., 1997). The activity of the SB system in zebrafish appears to be about an order of magnitude higher than that from two heterologous transposon systems, Tc3 from the nematode *C. elegans* (Raz et al., 1998) and *mariner* from insects (Fadool et al., 1998). An apparently active mobile element has been found in an albino medaka after it mutated a pigment gene (Inagaki et al., 1994; Koga and Hori, 1997). The responsible element, the *Tol2* transposon, encodes a transposase with excision activity but has yet to be tested for full autonomous transposition (Kawakami et al., 1998). Because a single nuclear localizing protein such as a transposase (Ivics et al., 1996) is sufficient to drag a DNA molecule into a nucleus (Zanta et al., 1999), the SB system offers a means of penetrating the nuclear membrane barrier as well as the chromosomal barrier to transgenesis. The SB system, and possibly the other transposons, should prove very useful for gene delivery and gene discovery in fish (Weinberg, 1998; Hackett et al., 1999).

Parallel with the development of the SB transposon, efforts were underway to investigate the abilities of internal ribosome entry sequences (IRESs) from mammalian picornaviruses (Jackson and Kaminski, 1995) to mediate internal initiation on cellular messages in fish. The IRES from the encephalomyocarditis virus (EMCV) of mice proved to be quite active in zebrafish embryos (Köster et al., 1996; Fahrenkrug et al., 1999). The IRES can be used for two purposes, tracking the effects of unknown gene products and finding new genes. In the first case, dicistronic

(also called bicistronic) vectors are made in which a gene-of-interest, which might have an unknown effect in a cell or tissue, is followed by an IRES and coupled reporter gene. The IRES directs expression of the reporter gene product everywhere that the first gene is expressed. In this way local phenotypic effects can be correlated with expression of a specific gene. In the second case, a DNA construct is made with a reporter gene behind an IRES but lacking either enhancers (called an enhancer-trap) or enhancers and a promoter (called a gene-trap) (von Melcher et al., 1990). As a result, the reporter gene can only be expressed when the enhancer-trap integrates in the vicinity of some cellular enhancer or the gene-trap integrates inside a transcriptional unit. In both cases the reporter genes are expressed under the control of natural regulators inside the genome. The traps also function as tags to allow easy isolation of the neighboring sequences (genes and promoters). IRES-containing gene-trap vectors have been effectively used in mice (Evans et al., 1997). In preliminary experiments using the SB transposon system to facilitate integration, the EMCV IRES plus a GFP gene acts as a powerful gene-trap in zebrafish to allow non-invasive, *in situ* detection of gene activity during early development (Clark, Hackett et al., unpub.).

The transposon system offers several subtle benefits as well as those already discussed. First, nonhomologous recombination of transgenic DNA often occurs as concatemers (Iyengar et al., 1996; Caldovic et al., 1999) which has been associated with silencing (Dorer, 1997; Garrick et al., 1998; Henikoff, 1998). Transposition inserts only single transposons at a time, even when the source might be several linked transposons. Thus, single copies of transposons should occur at a given transgenic locus. Moreover, as noted earlier, the two components of any transposon system are an active transposase and the DNA transposon that is mobilized. This binary requirement renders the SB system relatively safe when the source of transposase is a short-lived mRNA or protein rather than a gene. At this stage the SB system is in its infancy and will need more tweaking to find the optimal conditions for transgenesis.

2. New Elements for Genetic Constructs

Transcriptional regulatory elements can act over long distances in chromatin, up to nearly 100,000 base pairs. This raises important questions - what limits the range of enhancer activity or what keeps an enhancer from regulating a variety of genes. Because neighboring genes are individually regulated, there must be some type of border that insulates genes from one another. Active genes and their regulatory regions are often hypersensitive to DNases and extended into loops of decondensed chromatin. Limited digestion of chromatin with nucleases and extraction of histones has revealed a protein scaffold to which loops of DNA appear to be anchored. The sites on the genomic DNA that bind to proteins of the nuclear scaffold are called matrix attachment regions (MARs) and they may serve to define or establish independent domains of gene expression (Geyer, 1997; Pikaart et al., 1998). These sequences are called border elements,

specialized chromatin sequences (scs, Kellum and Schedl, 1991; Krebs and Dunaway, 1998), and attachment elements (A-elements, Bonifer et al., 1991, 1996). A-element sequences have been shown to have nuclear matrix-binding activity (Bonifer et al., 1991) and bestow position-independent expression of transgenes transgenic mice (Phi-Van et al., 1990; McKnight et al., 1992). That is, border elements can overcome or protect against position effects to allow proper regulated expression of a transgene if its integration site is in euchromatin.

Border elements appear to protect certain stretches of DNA from being transcriptionally silenced. Methylation appears to be involved although the general role of DNA methylation is still not clear. Methylation entered eukaryotic genomes at the evolutionary boundary between invertebrates and vertebrates (Tweedie et al., 1997). In fish, methylation patterns can vary depending on genotype (Martin and McGowan, 1995) and are important for proper embryonic development (Martin et al., 1999). Methylation may be a defense against the invasion of chromosomal DNA by foreign genes (Doefler, 1992) and/or transposable elements (Yoder et al., 1997). Up to 60 percent of vertebrate genomes, including those of fish (Izsvák et al., 1997; Ivics et al., 1999), may be derived from mobile repetitive sequences. Prohibiting methylation leads to transcription of some genes, including those interrupted by repetitive mobile elements (Bestor, 1998; Martienssen, 1998; O'Neill et al., 1998). However, Bird (1997) and Simmen et al. (1999) find no evidence that methylation has been adapted for regulation of repetitive elements and the genes in which they reside. The bottom line is that even though the nature and etiology of methylation are not understood, prokaryotic sequences should be removed from transgenic constructs.

Accordingly, insulator sequences from flies (scs sequences) and chickens (A-elements) were tested to determine whether they would function in zebrafish and confer not only position-independent expression, but also maintain expression levels after passage progeny. In these studies, *cat* genes driven by the carp β-actin promoter were tested in zebrafish for the uniformity of expression (Caldovic and Hackett, 1995). Although the efficiency of producing transgenic fish was low, about 2.4 percent, *cat* genes lacking either of the border elements gave non-uniform expression whereas either of the two border elements conferred position-independent expression that was maintained through three generations of fish. Moreover, within a factor of two in zebrafish, the expression per gene per cell at one, two and five days of development was constant for transgenes flanked with either *scs/scs'*- or A-elements (Caldovic et al., 1999). The uniformity of expression was all the more intriguing because concatemers of constructs inserted at a single site without apparent inactivation. Curiously, frequently what looked to be a single Mendelian locus of many transgenes turns out to be a number of tandem constructs interspersed with genomic DNA (Pawlowski and Somers, 1998; Aleström, personal communication). This data

suggests that the border elements can confer reliable levels of expression of a transgene in fish and protect the transgenes from being switched off as they are passed from generation to generation.

3. Fish Genetics and Bioinformatics

Transgenesis requires genes and information about how the genes are regulated and positioned. With haploid fish genomes varying from 2-5x10^9 base pairs containing 100,000 or more genes, the information that needs to be obtained and organized for easy retrieval is essential. Accordingly, efforts were made to find mutants in every gene necessary for proper development of a vertebrate. This was done using chemical mutagenesis of zebrafish genomes in two laboratories (for summaries of the results, see Driever et al., 1996; Haffter et al, 1996; Eisen, 1996). A second goal was to establish genetic maps in fish on which the above loci could be placed. These maps not only place specific genes, but also non-coding sequences that can be used as landmarks for mapping efforts (Nelson et al., 1989; Ziekiewicz et al., 1992) as well as phylogenetic relationships between species of fish (Izsvak et al., 1996, 1997, 1999).

The chemical mutagenesis screens provided insights into the numbers and effects of many genes required for the proper development of zebrafish. As an example, the screens indicated that there are close to 100 genes that regulate cardiovascular development and activity (Stanier et al., 1996; Weinstein et al., 1996; Chen et al., 1996). The activities of the encoded proteins can be dissected so that specific genes can be linked to specific physiological activities and correlated to defects related to human cardiac diseases (Fishman and Olson, 1997; Warren and Fishman, 1998). Dissection of other developmental pathways in fish began in the second wave. Mining the wealth of data from these screens will take several years and extend into the third wave of transgenesis in fish.

Zebrafish developmental genetics bloomed and looked as though it would meet the expectations of its founder, George Streisinger, who recognized its incredible value for examining the genetics vertebrate development (Streisinger et al., 1981). The most dramatic progress was in zebrafish where a comprehensive molecular genetic infrastructure was established. It began with construction of the zebrafish genome map (Postlethwait et al., 1994; 1999). The biology and molecular genetics of zebrafish have been reviewed in two volumes (Detrich et al., 1999a,b) and an updates are available from a, easily accessible multi-linked Internet web site (http://zfish.uoregon.edu/ZFIN/). Zebrafish-related biological and molecular reagents can be ordered through this site. The Institute for Genome Research (TIGR) maintains an organized zebrafish gene index (http://www.tigr.org/tdb/zgi/zgi.html).

Genome maps in another model fish, medaka, followed (Wada et al., 1995). Likewise, genetic maps of the genomes of several salmonid species are underway in the European Community.

The rate of acquisition of molecular genetics information about fish is increasing at a geometric rate. In 1995 and 1997 Gong et al. published the first surveys of 265 and 1084 expressed sequence tags (ESTs) for zebrafish. By the end of 1998 the number had increased about 10-fold to more than 10,000 zebrafish ESTs, all immediately accessible through the Internet (GenBank, 1998). The roles of many genes will be elucidated by various mutation strategies outlined above as well as two powerful techniques for rapidly marking the temporal and spatial expression patterns of genes. The first method will be to employ GFP containing gene-traps such as those made with the SB transposon system. This method will allow the identification of many unknown genes that are involved with the development of specific tissues. The second approach also involves using GFP for the same purposes, except here the GFP sequence is juxtaposed to a gene-of-interest. The periods and cellular/tissue patterns of expression can be monitored in situ by illuminating the developing fish with blue light (Peters et al., 1995). For instance, in zebrafish this method has been used to demonstrate the locations on cellular membranes of a putative connexin gene product (Essner et al., 1996).

4. Genetic vaccines

Diseases transmitted by microorganisms are becoming an ever greater problem in aquaculture and mariculture (Parfit, 1995). Genetic vaccination (Tang et al., 1992) has been proposed as a solution to these problems (Fjalestad et al., 1993; Mialhe et al., 1995; Ulmer et al., 1996). There are three ways in which immunity to disease-causing organisms has been induced in fish and they have been mostly concentrated on viruses. These are 1) immunization with a protein to establish an antigenic response, 2) direct immunization by muscle injection of a recombinant DNA that expresses an immunogenic protein, and 3) genetic transformation of fish genomes with a transgenic construct that expresses a viral protein.

The first fish vaccine from biotechnology was made against the VP2 capsid protein of the infectious pancreatic necrosis (IPN) viruses which represents the most serious problem in salmonid farming in Norway virus The VP2 protein was expressed and purified from *E. coli* (Christie, 1997; Lillehaug, 1997). The rhabdovirus of the Infectious Hematopoietic Necrosis Virus (IHNV), causes extensive mortality in northwest Pacific trout and salmon hatcheries (Wolf, 1988). The glycoprotein (G) and nucleoprotein (N) genes of IHNV were cloned and expressed in a baculovirus vector (Koerner and Leong, 1990) to provide the protein, which was used for passive inoculation to stimulate antibody production against the antigen and thereby achieve immunity (Engelking and Leong, 1989a,b). This procedure required dunking each fish individually in a tank containing the virus-free proteins. Clearly, genetic methods of immunization, as developed for model and domesticated animals (Raz et al., 1994; Robinson et al., 1997; Whalen, 1998), would require less labor if they were efficient in their delivery of pathogen resistance. These consist on direct injection of transgenic DNA into muscle tissue to induce immunity. DNA

vaccines can be produced in high quantities in a pure state for very low cost. Such vaccines are stable and therefore easy to store and ship. Directly injected transgenic DNA has been expressed in carp, tilapia, and *Xiphophorus* (Hansen et al., 1991; Rahman et al., 1992b; Anderson et al., 1996a,b; Gómez-Chiarri et al., 1996a; Tan and Chang, 1997; Schulte et al., 1998), suggesting that this method of somatic DNA transfer can be useful for vaccination.

The potential of DNA vaccines have been tested in experimental conditions and have protected rainbow trout against bacterial kidney disease (Gómez-Chiarri et al., 1996b) and viral hemorragic septicemia (Heppell et al., 1998). These vaccines are not yet approved for veterinary use in aquaculture. The potential of RNA-based vaccines is also alternatively tested, either as naked molecules encoding the VP2 gene as protective against the (IPN), or packaged in suicide virus particles or empty capsids for easy delivery (Vaughan et al., 1998).

Muscle injection may be straightforward, but it does not provide the advantage of germline passage of immunity as would be the introduction of the recombinant DNA during early embryogenesis. Having shown the potential of the IHNV viral proteins to elicit immunity, the next step was to use germ-line transgenesis to immunize fish and their offspring, which apparently worked (Leong et al., 1997). When a CMV/G-protein gene construct, with or without a CMV/N-protein gene expression construct, was injected into rainbow trout embryos, survival rates of transgenic fish challenged at a later time to IHNV were nearly three times higher than controls (Anderson et al., 1996a).

Diagnosis of fish pathogens (Austin, 1998) and detection of protective antigens should parallel the development of new methods for genetic immunization. In the long run, genetic immunization may be far more valuable to the aquaculture industry than any other productive trait. Environmentally and socially this type of genetic engineering in fish may be more acceptable than conferring new phenotypes related to enhanced-growth.

5. Accomplishments during the Second Wave

The major accomplishments of transgenics in fish in the second wave were primarily in model fish systems. The success in making growth-enhanced commercial fish in the first wave was tainted by difficulties in distributing them, in part due to concerns over possible escapees into the environment. The development of genetically immunized, disease-resistant fish may be an exception when they come on line. Political and environmental concerns do not apply to model fish systems, which provided new sets of genetic tools for application to aquaculture and mariculture.

D. Fish Genetic Engineering: The Third Wave

Few of the genes that are involved in growth and development have been identified although the zebrafish screens discussed earlier indicate that there are many. In fish, as with all other animals of commercial value, the interactions of the myriad of cellular proteins in differentiated cells is poorly understood. This is because most of the traits that are important for improvement of commercial fish are polygenic. The result is that genetic engineers currently cannot achieve the types of modifications that are obtainable from breeding programs. To overcome this problem, many more genes in piscine genomes must be identified and characterized with respect to their temporal and spatial patterns of expression. This information is necessary to link genes with specific traits and physiological conditions. Moreover, the consequences in specific tissues of over-expression and under-expression of physiologically important genes must be understood.

In order to obtain this type of information, a variety of genetic procedures that have been employed in other organisms, primarily prokaryotes and simple eukaryotes, must be developed for fish. These include methods for insertional mutagenesis, such as gene-tagging, gene inactivation, and yet more efficient procedures for producing transgenic animals. Some trends that we see developing are discussed below.

1. Genomics

Precision genetic engineering in fish will be a major goal in the third wave. By this we mean that the effects of the transgenes will be more localized and specific than those such as the growth hormones. There are two types of genomic studies; those that look to identify and map genes (what we will call classical genomics) and the effects of alteration of gene expression in order to gain some understanding of the function and importance of a given gene (what we will call physiological genomics).

a. Classical Genomics

The goals and techniques of classical genomics were described earlier in the section on Genetics and Bioinformatics. As an example of what we expect from genomics in the future, we look at two trends that relate to one of the foremost goals of genetic engineering in fish -- improved growth rates accompanied by increased feed conversion rates.

Recent evidence suggests that precision transgenics may be more feasible in fish than in other animals because some fish genomes have apparently undergone partial or complete tetraploidization (Postlethwait et al., 1999; Wittbrodt et al., 1998). A major consequence appears to be that a gene in mammals that has two or more functions may have these functions split between genes. A good example is exemplified by a gene that may be useful in aquaculture, *c-ski*. C-*ski* is the cellular homologue of the oncogene v-*ski* that was isolated from an avian leukosis virus (Stavnezer et al., 1986). Chick embryo cells and quail embryo cells transfected with c-*ski* undergo transformation to myoblasts and differentiate into myotubes (Namciu et al., 1995).

Transgenic mice with a truncated form of chicken c-*ski* show selective skeletal muscle hypertrophy, making them look like *supermice* with extraordinary musculature (Sutrave et al., 1990). However, the c-Ski protein is a transcriptional factor (Nomura et al., 1999) that is highly expressed in the central nervous system (Lyons et al., 1994); knockout mutants in mice die because the neural tube fails to close during development (Berk et al., 1997). Thus, in mammals, c-*ski* has a wider role than just in muscle development. In zebrafish the c-*ski* is duplicated and it appears that the c-*ski*A and c-*ski*B genes may have more precise roles in muscle compared with central nervous system development (Kaufman et al. 1999). If this turns out to be the case, then it may be possible to induce selective growth in fish tissues by adding only one of the c-*ski* genes to fish chromosomes. Fish with greater musculature might be attractive for aquaculture where the fish could gain extra value. The bottom line is that identification of genes in fish may hold greater promise for genetic engineers than similar endeavors in farm animals. And, as an additional bonus, the same studies may provide developmental insights that are not available using mammalian and avian species.

Growth factors with greater specificity are one approach to engineering fish with greater commercial value. An alternative approach is to attack the problem from the other end, to deregulate limitations on growth of muscle tissue. Although size differences between animals are so very obvious, little is known about why this is (reviewed in McKnight, 1997; Conlon and Raff, 1999). However, deregulation of muscle growth can be achieved by mutation in land animals (*e.g.,* Grobet et al., 1998) and the gene has been identified -- myostatin (McPherron and Lee, 1997; McPherron et al., 1997). Thus, interruption of this gene may produce the same phenotypes as transgenesis with additional growth hormone-like genes. This raises the question of how the flow of genetic information can be disrupted selectively in fish.

The third wave will see functional genomics extend from model fish to fish of commercial importance. The saturation screens accomplished in zebrafish would be prohibitively expensive and too time consuming if applied to large fish. The linkages of genes are often conserved over short stretches, termed synteny, so that maps from one organism can help in the placement of genes in another. Moreover, several alternative genetic procedures, already used for mapping genomes of domesticated land animals, are being employed in aquaculture. These strategies are designed to identify genetic loci containing several genes, which increase the value of the animal/fish. Because the genetic resolution of these procedures is lower than mapping genes one by one, identifying such loci is faster and cheaper than precision mapping. The process is called marker-assisted selection (MAS). As an example, in the United States, catfish account for more than 50 percent of all aquacultural production. Accordingly, major efforts were initiated to develop maps of the catfish genome for MAS in the breeding of catfish. Essentially, various traits can be linked to chromosomal markers, also known as economic trait loci (ETL). These markers

often are not genes themselves, but rather simple sequence repeats (microsatellites, see O'Connell and Wrist, 1997), random amplified polymorphic DNA fragments (RAPDs), or amplified fragment length polymorphism fragments (AFLPs). The linkage information allows application of MAS and eventual cloning of the genes responsible for the performance traits. The correlation of chromosomal sites with phenotypic characteristics (*e.g.*, ETL) is called quantitative trait locus (QTL) mapping. As the density of the marker sequences in a genome grows, the specificity of mapping genes increases, which is necessary for marker-assisted selection in breeding. Large numbers of RAPD (Liu et al. 1998a) and AFLP (Liu et al., 1998b, 1999) markers continue to be developed to establish better genetic and QTL maps in catfish as a foundation for MAS (Liu and Dunham, 1998). The same work will be applied to commercial fish important to the European Community and Asiatic countries.

b. Physiological Genomics

Finding the identities and map positions of genes is becoming easier to do. As noted earlier, entries into the genetic databases are increasing at geometric rates and is likely to do so for several years. Every year these procedures become easier, faster, and cheaper. Yet, for all of the information that is gathered, geneticists and fish breeders have little information as to what the genes actually do. For this, mutational analysis is important. However, coupling the effects of mutations with specific genes, a field known as functional genomics or physiological genomics, is difficult in vertebrates because of their enormous genomes. Gene-traps and enhancer-traps, described earlier in the section on transposons, offer one powerful method to rapidly identify mutations that cause specific phenotypic changes in fish. The vectors and methods for using them are being developed for the third wave.

2. Genetic Inactivation and Site-specific Mutation

There are two ways of interrupting the flow of genetic information in cells. The first is to mutate selectively a gene. The second is to interrupt either processing or translation of its mRNA. We will look at methods for doing this in reverse order.

a. Inactivation of Expression

In addition to bringing in new genes whose expression confers new traits, transgenic constructs may be designed to create *loss-of-function* mutants. The most direct way is to transcribe the complementary strand of the gene-of-interest, *i.e.* transcribe the gene in the opposite orientation, to make an antisense transcript that could inhibit normal expression (Izant and Weintraub, 1985). This procedure does not always work and the causes of failure are not clear. Some problems may be the distribution of the target mRNA into a different cellular compartment than its antisense sequence, differential stabilities of the mRNA and antisense complement, and RNA folding of either the antisense or mRNA molecule so as to block effective hybridization. In fact, there are several mechanisms through which anti-sense RNA may exert its

effects (Baserga and Denhardt, 1992). Nevertheless, the procedure is so easily understood that has been commonly used in the beginning.

Surprisingly, there are few reports of antisense use in fish, which may be an indication that it is not very effective in these animals. In zebrafish antisense RNA has been used to investigate the roles of two proteins (connexin 10 and the *forkhead* gene product) during development (Barabino et al., 1997; and Roth et al., 1999). One potential problem is that antisense RNAs are naturally made, and some of them have the potential of encoding proteins. This is the case with the Na/phosphate co-transporter gene in winter flounder fish where both sense and antisense transcripts can be found (Huelseweh et al., 1998).

A similar strategy is the use of catalytic RNA, also known as ribozymes (Haseloff and Gerlach, 1988). The method employs the ability of certain conformations of RNA to bind to other RNA sequences and, via a transesterification reaction, cleave the target RNA. The minimal catalytic sequence is under 50 nucleotides and quite active (McCall et al., 1992). The result is an RNA that can seek out and cut specific mRNAs at a specific site and thereby achieve the same effect as antisense RNA. The catalytic RNAs, called ribozymes, act as enzymes and thus can attack a much larger number of mRNA targets so that a large excess of the ribozyme may not be required as is the case with antisense RNA. Ribozyme-knockout of gene expression has been attempted in zebrafish, where it was able to reduce, but not eliminate, expression of the *no tail* (*ntl* or *brachyury*) gene (Xie et al., 1997). The disadvantage of catalytic RNA is the complexity in designing the killer transcript -- the folded structure of the target mRNA should be known to ensure binding of the ribozyme.

Recently a most intriguing method has been successfully employed to silence gene expression in animals, double-stranded interfering RNA (dsRNAi). The injection of dsRNAi into nematodes (Fire et al., 1998) or Drosophila (Kennerdell and Carthew, 1998) completely abolishes synthesis of the gene product. dsRNAi must contain some portion of the final mRNA sequences to be functional but intronic sequences have no effect in the dsRNAi. The mechanism by which dsRNAi operates is unknown, but it is suspected that cells employ such activity normally (Montgomery and Fire, 1998).

The advantage of these strategies is that they are fairly easy to use in terms of their construction and delivery to single cells of very small animals. The disadvantage is that they may not completely abrogate gene expression in every cell and the effects certainly cannot be passed to progeny. For that, modification of the genome is necessary. This may explain the paucity of reports using any of these methods for interrupting the flow of genetic information in fish.

b. Site-specific mutagenesis

Gene-targeting to inactivate genes, by selecting for very low rates of homologous recombination in embryonic stem (ES) cells (Mansour et al., 1988; Capecchi, 1989) has been a

boon to functional genomics in mice. Heretofore the procedure has been relatively inefficient and used only on embryonic stem (ES) cells in culture which are then injected into the developing animal (*e.g.*, Thomas et al., 1992). ES cell technology has only been successfully realized in mice, although there are attempts to develop the technology in fish (discussed in the following section). In the meantime, two other techniques are being examined that may prove to be more versatile in fish. These techniques are based on the activities of DNA-repair enzymes.

DNA molecules in animal cells are examined for mismatched base pairs. When problems are found repairs are made by enzymes that apparently can use RNA in a homologous recombination-based repair process (Kotani and Kmiec, 1994a, b). This led to the testing of covalently coupled DNA-RNA chimeric molecules that can selectively insert single base-pair changes at defined loci in chromosomal DNA (Alexeev and Yoon, 1998; Kren et al., 1998). Moreover, the constructs can be targeted to specific cells such as those in liver (Kren et al., 1997) using appropriate DNA-condensing agents and liposomal mixtures (Bandyopadhyay et al., 1998). A related procedure makes direct use of *E. coli* RecA-coated single-stranded DNA molecules which seek out their complements in genomic DNA and direct homologous recombination (Sena and Zarling, 1993; Pati et al., 1997). This procedure has the potential of introducing changes larger than a single base-pair, the current limitation on the chimeric RNA-DNA hybrid molecules. These procedures have yet to be tried in fish. However, based on the success of microinjection in fish, delivery of transgenic DNA fragments that can seek out specific sites for homologous recombination should be possible to achieve at high efficiency.

3. ES-cell technology

As noted earlier, genetic engineering of fish by introducing transgenes directly into oocytes or early embryos is extremely labor intensive and inefficient. The screening process involving whole animals is expensive and timing consuming. Two methods have been developed to circumvent these difficulties, Embryonic Stem (ES) cell technology and nuclear transfer. Both fall under the general term somatic cell transfer because the transgenic DNA is delivered with an entire genome rather than on a single gene basis. Nevertheless, both methodologies are to enhance transgenesis and the amplification of certain genotypes. It should be noted that these methods were developed for land vertebrates where embryos are scarce and must be implanted following transgenesis into recipient mothers for gestation. Transgenesis in fish does not suffer these disadvantages. In this section we briefly review ES cell technology and nuclear transfer technology in the following section.

ES cell technology was developed to improve the efficiency of making site-specific mutations in animal genomes. ES cells derive from cultured cells and are able to keep their totipotency to differentiate into virtually any cell-type when transplanted into an embryo. For transgenesis, the important point is that ES cells can populate the recipient's germ line. The ES

cell system permits pre-selection of defined mutations before introduction into animals. Thus, it can be used to remove all unwanted genetic background events that normally accompany mutagenesis. The resulting animals with defined mutations can be examined for phenotypic effects that may be of particular interest in aquaculture. ES cell technology also can be used to study genes involved in early development. Unfortunately, ES-cell technology has been fully developed only in mice; no reproducible germline chimeras have so far been reported in any other animal. Nevertheless, fish are especially attractive for developing ES cell technology because of the use to which it can be put in commercial species and the experimental advantages of working with fish embryos to answer basic questions about development.

There have been many attempts to derive ES-cells by culturing cells from blastocyst of zebrafish and medaka (Collodi et al., 1992; Wakamatsu et al. 1994; Sun et al. 1995a). The results of this early work defined some necessary parameters for obtaining and growing cells. However, in all cases only short-term cultures were achieved (Sun et al., 1995b). This changed with the long-term embryonic cell culture of medaka cells in an apparently undifferentiated state without feeder layer cells (Hong and Schartl, 1996a). The same conditions have been adopted for developing ES cells from the marine species seabream (*Sparus aurata*) (Béjar et al, 1998). The success in obtaining long-term putative ES cells in medaka and seabream suggests that the conditions might carry over to other fish species, although it is thought that successful derivation of ES cells may depend in part on the genetic background (Simpson et al., 1997; Alvarez et al., unpub.).

A useful way to evaluate and predict the quality of the putative ES cells in terms of their capacity to contribute in formation of chimeras is to perform a set of *in vitro* tests, which in mice are effective for pre-selecting ES cell strains. In fish two cells lines have been isolated with ES cell potential First is the MES1 line from medaka, which has been maintained for more than 140 passages, over more than two years, and has been extensively characterized *in vitro* (Hong et al., 1996a,b). The second is the SaBE1 cell culture from seabream, which has been cultured over four years and more than 40 passages (Béjar et al., 1999). Both of lines show the typical morphology of mice ES cells: small size, sparse cytoplasm and large nuclei, normal and stable karyotypes, high alkaline phosphatase activity and the ability to form compact colonies. Moreover, all descendants from single colonies of MES1 cells were able to produce ES-like cells as well as differentiate to the same type as the parental line (Hong et al., 1996 b). A stable karyotype is important; there are indications that chromosome changes are responsible for the loss of totipotency in some cultures (Longo et al., 1997). Important features of ES-cells are that they can be cryopreserved, transfected, and retain homologous recombination activity. MES1 cells have all three properties and **SaBE1** cells have at least the first two (Hong et al., 1998 and Béjar et al., 1999).

The ultimate test for putative ES cells is to produce chimeric fish composed of cells from the original embryo and the ES-donor cells. Direct injection of blastomeres into recipient embryos has produced germ-line cells containing genomes from both sources in rainbow trout (Nilsson and Cloud, 1992), zebrafish (Lin et al., 1992) and medaka (Wakamatsu et al., 1993). However, when cultured embryonic cells have been used for chimera formation the results have been mixed. Zebrafish chimeras did not survive (Speksnijder et al., 1997). In medaka, donor cells contributed to numerous organs derived from all three embryonic layers, the first evidence of somatic chimeras (Hong et al., 1998). The chimeras have been grown to adulthood and undergone sexual maturation. The lower production rate of chimeras obtained with ES-like cultures when compared with that obtained with blastomeres may partly be due to differences between donor and recipients in their morphological and physiological traits such as cell size, transcriptional activity and cell cycle duration (Hong et al., 1998). Genetic compatibility between donor and host strains appears to be very important.

In the third wave of fish transgenesis more research is required to extend ES-cell technology. This will involve the following:

a) Testing different genetic strains for their adaptive abilities to culture conditions while maintaining their totipotency. Because of the apparent need for genetic compatibility between donor and recipient, many different strains of fish will have to be tested. This task is less difficult in model species (although chimeric zebrafish have yet to be raised) than for commercial species. Mapping genomes and developing genetic markers in commercial fish, as already described for catfish, are essential but will take considerable time and effort.

b) Development of methods to overcome physiological differences (*e.g.*, transcriptional activity, cell cycle length, etc.) between donor ES cells and those of the recipient blastula-stage embryos.

c) Alternatively, isolation of sufficient totipotent cells from blastocysts in order to produce germ line chimeras when permanent lines can not be established.

4. Nuclear Transfer technology

The world may have been shocked when it woke up one morning to hear that a mammal had been cloned. The nucleus of a cell, which had been isolated from one strain of sheep and tissue-cultured *in vitro*, provided the genetic instructions for development of a living animal in an enucleated oocyte of another sheep (Campbell et al., 1996). The ultimate test of the method was the demonstration that the cloned sheep could grow to adulthood and reproduce in the natural way (Wilmut et al., 1997). Thus, the era of animal cloning had arrived. Or so the popular press thought. Actually, the technique of transferring a nucleus from one cell to another began more than 100 years ago (Rauber, 1886 - who noted "painful responses" by the recipient cell to the transferred nucleus) and more systematically a half-century later with the first transfer of fish

nuclei (Tung et al., 1945). The ability of transferred nuclei to direct successful development in fish was achieved about twenty years later (Tung et al., 1965). In contrast, successful nuclear transfer in mammals took almost 20 more years to achieve than in fish (McGrath and Solter, 1983), and the ability to achieve complete development following nuclear transfer (cloning) took another 13 years, culminating in the results of Wilmut and Campbell. The experiments in fish, amphibians, and mammals demonstrated that the developmental potential of nuclei becomes restricted as embryogenesis progresses (Gurdon 1986). The key to achieving the final goal of complete cloning of an animal was an appreciation that the cell division cycles of the donated nucleus and the recipient oocyte had to be coordinated so that the nuclear programming matched the cell cycles of the developing embryo. Here we review the progress on "fish cloning".

In fish, oocytes arrested at meiotic metaphase II are usually used as host cytoplasts. Once the chorion is mechanically removed, the oocytes are activated via contact with water or by fertilization and the eggs go through meiosis II, when the second polar body is microscopically visible. At this time the nucleus, which is located just beneath the polar body, can be removed (along with the polar body) by microsurgury using a fine glass needle. This is the most difficult step because inevitably some cytoplasm, which may be essential for early development, may adhere to or come along with the nucleus. Consequently, it is desirable to use noninvasive enucleation methods such as irradiation with ultraviolet (UV) light, or irradiation with gamma or X-rays. These methods have been successfully used in land animals, but not as yet in fish.

Donor nuclei are usually prepared from blastula-stage embryos. Single blastomeres, which have been dissociated from these embryos, are swollen in a hypotonic buffer and aspirated into a glass needle, which causes them to burst and and liberate their nuclei. A single nucleus can then be introduced into an enucleated egg at or near the position of the original nucleus. Such "re-nucleated" eggs then are incubated in a balanced salt solution until hatching (Hong et al., 1996c).

For transgenesis, blastomeres are not ideal donors of nuclei because selecting for specific changes is not practical. Consequently, there have been attempts to use nuclei from cultured cells (personal communications from Y. Hong and J. Béjar). Chen et al. (1986) have been able to transfer nuclei of cultured cells into enucleated oocytes of goldfish to obtain a couple of adult fish. The fish were sterile, probably due to aneuploidy in the transferred nuclei. Another source of donor nuclei may be primordial germ cells (PGCs) from more advanced embryos, which at least in mice maintain the ability to promote development following nuclear transfer (Tsunoda et al. 1989). However, little is known about PGCs in fish. Once donor nuclei are immersed in new cytoplasms, they undergo a series of morphological and physiological changes known as nuclear reprogramming or remodeling. The more synchronized the cells cycles in the nuclei and cytoplasms, the more successful the union of the cellular compartments will be. However, one problem with using cultured fish cells as nuclear donors is that their cell divisions are slower than

those of early embryos. This problem of non-synchrony between alien nuclei and recipient cytoplasms might be overcome in fish by using serial nuclear transfer experiments or using less mature oocytes to allow the nuclei more time to adapt to the oocyte schedule.

Reprogramming of fish oocytes has largely been done in China to study nucleo-cytoplasmic interactions as well as to test the limitations of nuclear transfer for genetic improvement in aquaculture (Yan, 1998). Efforts have included attempting intra-species, intra-genera, intra-order, and even mammal-fish matches in fish oocytes (Yan, 1989; Yan et al., 1990, 1991; Zhang et al., 1990). Matched nuclei and oocytes from the same species and genera produced a few fertile adults; but no animals could develop beyond the fry stage with more mixed matches. An excellent compilation of the extensive nuclear transfer experiments have performed in various cyprinid fishes has been reviewed in Schartl et al., (1998).

The big question is whether perfecting these procedures of moving genomes, and other techniques such as transfer of an artificial chromosome (Harrington et al., 1997), will turn out to be necessary in fish. Somatic genome transfer by ES cell technology has been most useful for analyzing the effects of selectively inactivating one or both alleles of a gene and nuclear transplantation is most useful for amplifying particular transgenic genotypes that are practically impossible to obtain by "routine" genetic engineering. This applies to land vertebrates but it may not apply to fish, where these techniques still have not been brought on-line. In fish, these strategies may prove to be more arduous than making changes, in many embryos using the transgenic procedures discussed earlier, and selecting for those that are desirable. When gene knock-outs (inactivation of a gene) or gene "knock-ins" (when a gene is selectively placed into a certain chromosomal locus) are desired, other recombination-based techniques under development may be easier and have higher yields. That is what will make the third wave of genetic engineering in fish so exciting, there are many possibilities waiting to be developed and exploited.

E. Public Policy Issues Concerning Genetic Engineering in Fish

The goals of making transgenic fish seemed impeccable – to feed the world. However, soon after the first wave of fish transgenesis was underway, questions were raised about the environmental consequences of transgenic animals that might escape their confines (*e.g.*, Bruggemann, 1993). This concern was enhanced in the case of fish, animals that schooled in large numbers, could not be easily tracked and had access to every part of the globe via waterways and oceans (Kapuscinski and Hallerman, 1990, 1991; Devlin et al., 1992). Many of the concerns were based on infamous invasions of aggressive alien species of plants and animals brought by humans into naïve ecosystems.

The debates of whether "designer" fish should be allowed out of enclosed laboratories resolved into two issues. First, are transgenic species in which a single gene is altered equivalent

to an alien species? A related question is never asked. What degree of difference in a single gene qualifies an animal as being an alien rather than just a mutant? This is a critical question because virtually every vertebrate animal differs from all others in terms of mutated genes. It would seem that human-mediated genetic engineering defines a mutation as alien even though the equivalent genetic change occurring naturally would be considered an example of natural diversity, something which is appreciated and desirable. Another aspect of the issue has been the claim that humans can move genes between species, genera, families, etc. whereas natural movement of genes is more limited. However, recent investigations have shown that there have been, and probably continue to be, natural massive exchanges between phylogenetically very distant organisms (e.g., Doolittle, 1997; Jain et al., 1999). This appears so in fish as well (Izsvák et al., 1995, 1997). Thus, nature has done, and continues to do, what genetic engineers try to do; it is the time-scale and the selection processes that differ.

The second issue is how to determine the level of danger that escapee transgenic fish pose. The assumption was that until transgenic fish were proved to be safe, there should be some type of regulation on their dispersal (Hallerman, and Kapuscinski, 1992). However, one lone voice has noted that from a population genetics view, there appears to be very little inherent danger in engineered fish (Knibb, 1996). This is based on many findings that when organisms are unbalanced in some trait, their fitness decreases compared with that of normal, wild type individuals. Most genetically engineered fish have extreme phenotypes that require more care than wild populations. That means that the transferred gene would most likely act in the same way as a deleterious allele, which would likely be eliminated by natural selection (Knibb, 1996). One phenomenon has been noticed, but has not been scientifically evaluated, that of increased mortality of some growth-enhanced fish (salmon, Hew, per. comm.; our observations with northern pike and trout), suggesting that unbalanced levels of growth hormone may reduce fitness in transgenic fish. If so this would have significance in terms of fears of accidental escape of genetically engineered *superfish*.

A general principle of some ecological schools seems to adhere the statement that "this is the best of all possible worlds." (Voltaire, 1759). Thus, the introduction of a human-made genotype into nature might result in a change, which by this principle would be bad. The dilemma of many governmental regulatory agencies, which face this problem, is how to test the danger of engineered fish without either releasing them or conducting an endless number of highly controlled experiments that would have little real-world significance. Nevertheless, safety evaluations are proceeding with the first report of the behavior of growth-enhanced tilapia, which have a lower feeding motivation and dominance status compared with wild type fish (Guillen et al., 1999). Many developed countries put moratoria on the distribution of genetically engineered fish despite the lack of any evidence that they threaten the environment. The situation has been

aggravated by the developments in the areas of somatic gene transfer, nuclear transfer and cloning of animals. These advances in cellular engineering have induced discussions everywhere about the ethics of cloning and its consequences (*e.g.*, Mench, 1999). Almost all of these discussions, like those regarding release of genetically modified animals, revolve around philosophical, religious, and political ideas for which science does not have answers. Since nearly all of the research done by genetic engineers is accomplished with tax revenues, governments listen. To their credit, most scientific agencies in most countries have taken the long view and continued to support the research that has taken us to this point.

F. Summary: Genetic Engineering in Fish in the 21st Century

Development of new molecular genetic techniques for genetic engineering of fish will permit the following: 1) Efficient and targeted insertion of genetic material into fish chromosomes. 2) Identification, isolation, and characterization of genes, and their regulatory elements, that are involved in production-related traits. 3) Use of molecular markers for quantitative trait loci (QTL) analysis that will assist fish geneticists in the breeding of new strains. 4) Site-specific gene inactivation for obtaining stocks with specific inactivated genes, which could be useful for producing sterile transgenic fish; broodstock with inactivated genes could be mated to produce sterile offspring for either biological containment or for maximizing growth rates in aquacultured fish. The challenge remains -- to produce more fish at lower environmental and economic costs to feed the world.

G. ACKNOWLEDGEMENTS

We are grateful for the considerable help from colleagues in our labs, including Zhanjiang (John) Liu, Boaz Moav, Zoltan Ivics, Zsuzsa Izsvák, Luba Caldovic, Karl Clark, Deanna Mohn, Jeff Essner, Chris Kaufman, Julia Béjar, Sonia García-Pozo, J. Antonio Alarcon and Sergio Oliva. We appreciate the openness and discussions of our many colleagues, especially Drs. S. Ekker, M. Schartl, Y. Hong, N. Maclean, R. Devlin, R. Dunham, C. Hew, M. Gómez-Chiarri, and W. Knibb for discussions and sharing their results, sometimes prior to publication. We greatly appreciate the comments and corrections made by Zhanjiang (John) Liu (Auburn University). We apologize to the many authors of works in this area that we have failed to include in this review (let us know so that it will not happen again). Our laboratories are supported by grants from NIH, USDA, and the Minnesota Sea Grant program (publication JR 467) to PBH, and the EC and the CICYT to MCA.

H. References

Adams, M.D., et. al. (1992). Sequence identification of 2,375 human brain genes. Nature 355: 632-634.

Agellon, L.B., C.J. Emery, J.M. Jones, S.L. Davies, A.D. Dingle, and T.T. Chen. (1988). Promotion of rapid growth of rainbow trout (*Salmo gairdneri*) by a recombinant fish growth hormone. Can. J. Fish. Aquat. Sci. 45: 146-151.

Akasaka, K., A. Nishimura, K. Hijikata, Y. Iuchi, J. Morokuma, M. Takahashi, H. Morikawa and H. Shimada (1995). Introduction of DNA into sea urchin eggs by particle gun. Mol. Mar. Biol. Biotech. 4: 255-261.

Alam, M.S., A. Popplewell and N. Maclean (1996). Germ line transmission and expression of a *lacZ* containing transgene in tilapia (*Oreochromis niloticus*). Transgenic Res. 5: 87-95.

Aleström, P., G. Kisen, H. Klungland, and O. Anderson (1992). Fish gonadotropin-releasing hormone gene and molecular approaches for control of sexual maturation: development of a transgenic fish model. Mol. Mar. Biol. Biotech. 1: 376-379.

Alexeev, V. and K. Yoon (1998). Stable and inheritable changes in genotype and phenotype of albino melanocytes induced by an RNA-DNA oligonucleotide. Nature Biotech. 16: 1343-1346.

Allende, M. L., A. Amsterdam, T. Becker, K. Kawakami, N. Gaiano and N. Hopkins (1996). Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. Genes Dev 10: 3141-3155.

Amsterdam, A., S. Lin and N. Hopkins (1995). The *Aequorea victoria* green fluorescent protein can be used as a reporter in live zebrafish embryos. Dev Biol 171: 123-129.

Amsterdam, A., S. Lin, L.G. Moss and N. Hopkins (1996). Requirements for green fluorescent protein detection in transgenic zebrafish embryos. Gene 173: 99-103.

Anderson, E.D., D.V. Mourich, S.C. Fahrenkrug, S. LaPatra, J. Shepard, and J.C. Leong. (1996a). Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Mol. Mar. Biol. Biotech. 5: 114-122.

Anderson, E.D., D.V. Mourich and J.C. Leong. (1996b). Gene expression in rainbow trout (*Oncorhynchus mykiss*) following intramuscular injection of DNA. Mol. Mar. Biol. Biotech. 5: 105-113.

Arezzo, F. (1989). Sea urchin sperm as a vector of foreign genetic information. Cell. Biol. Int. Rep. 13: 391-404.

Atkinson, P.W., E.R. Hines, S. Beaton, K.I. Matthaei, K.C. Reed, and M.P. Bradley (1991). Association of exogenous DNA with cattle and insect spermatozoa in vitro. Mol. Reprod. Dev. 29: 1-5.

Austin, B. (1998). Biotechnology and diagnosis and control of fish pathogens. J. Mar. Biotech. 6: 1-2.

Bandyopadhyay, P., B.T. Kren, X. Ma and C.J. Steer (1998). Enhanced gene transfer into HuH-7 cells and primary rat hepatocytes using targeted liposomes and polyethylenimine. BioTechniques 25: 282-292.

Barabino, S.M., F. Spada, F. Cotelli and E. Boncinelli (1997). Inactivation of the zebrafish homologue of Chx10 by antisense oligonucleotides causes eye malformations similar to the ocular retardation phenotype. Mech. Dev. 63: 133-143.

Baserga, R. and D.T. Denhardt (1992). Antisense Strategies. Annals N.Y. Acad. Sci. 660. Ny, NY. Pp. 353.

Bayer, T.A. and J.A. Campos-Ortega (1992). A transgene containing *lacZ* is expressed in primary sensory neurons in zebrafish. Development 115: 421-426.

Bearzotti, M., E. perrot, C. Michard-Vanhee, G. Jolivet, J. Attal, M-C. Theron, C. Puissant, M. Dreano, J.J. Kopchick, R. Powell, G. Gannon, L-M. Houdebine, and D. Chourrout (1992). Gene expression following transfection of fish cells. J. Biotech. 26: 315-325.

Béjar, J., Y. Hong and M.C. Alvarez (1998). Development of ES cells from *Sparus aurata* embryos: Preliminary results. CIHEAM Journal Cahiers Options Mediterranéens 34: 131-139.

Béjar, J., Y. Hong and M.C. Alvarez (1999). Towards obtaining ES cells in the marine fish species *Sparus aurata*; Multipassage maintenance, characterisation and transfection. Genetic Analysis Biomolecular Engineering, (in press).

Benyumov, A.O., G.N. Enikolopov, V.A. Barmintsev, I.A. Zelenina, L.A. Sleptsova, Y.K. Doronin, V.A. Golichenkov, M.A. Grashuck, G.P. Georgiev, P.M. Rubtov, K.G. Skyrabin and A.A. Baev (1989). Integration and expression of the human somatotropic hormone gene in a bony fish. Genetika 25: 24-35.

Berk, M., S.Y. Desai, H.C. Heyman and C. Colmenares (1997). Mice lacking the ski proto-oncogene have defects in neurulation, craiofacial patterning, and skeletal muscle development. Genes Dev. 11: 2029-2039.

Bestor, T.H. (1998). Methylation meets acetylation. Nature 393: 311-312.

Bétancourt, O.H., J. Attal, M.C. Théron, C. Pussant, and L.M. Houdebine (1993). Efficiency of introns from various origins in fish cells. Mol. Mar. Biol. Biotech. 2: 181-188.

Bird, A. (1997). Does DNA methylation control transposition of selfish elements in the germline? Trends Genet. 13: 469-470.

Blackwood, E.M. and J.T. Kadonga (1998). Going the distance: A current view of enhancer action. Science 281: 60-63.

Bonifer, C., A. Hecht, H. Saueressig, D.M. Winter and A.E. Sippel (1991). Dynamic chromatin: the regulatory domain organization of eukaryotic gene loci. J. Cell. Biochem. 47: 99-108.

Bonifer, C., Huber, M.C., Faust, N. and Sippel, A.E. (**1996**). Regulation of the chicken lysozyme locus in transgenic mice. Crit Rev Eukaryot Gene Expr 6: 285-297.

Brem, G., B. Brenig, G. Horstgen-Schwark and E.L. Winnacker (1988). Gene transfer in Tilapia (*Oreochromis niloticus*). Aquaculture 68: 209-219.

Brinster, R.L., J.M. Allen, R.R. Behringer, R.E. Gelinas and R.D. Palmiter (1988). Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA 85: 836-840.

Brinster, R.L., E.P. Sandgren, R.R. Behringer, and R.D. Palmiter (1989). No simple solution for making transgenic mice. Cell 59: 239-241.

Bruggemann, E.P. (1993). Environmental safety issues for genetically modified animals. J. Anim. Sci. 71: 47-50.

Buono, R.J. and P.J. Linzer (1992). Transient expression of RSVCAT in transgenic zebrafish made by electroporation. Mol. Mar. Biol. Biotech. 1: 271-275.

Burns, J.C., T. Friedmann, W. Driever, M. Burrascano and J.K. Yee (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci. USA 90: 8033-8037.

Caldovic, L. and P.B. Hackett (1995). Development of position-independent expression vectors and their transfer into transgenic fish. Mol. Mar. Biol. Biotech. 4: 51-61.

Caldovic, L., D. Agalliu and P.B. Hackett (1999). Position-independent expression and germline transmission of transgenic DNA in zebrafish. Trans. Res. 8: 321-334.

Campbell, K.H.S., J. McWhir, W.A. Ritchie and I. Wilmut (1996). Sheep cloned by nuclear transfer from a cultured cell line. Nature 380: 64-66.

Capecchi, M.R. (1989) Altering the genome by homologous recombination. Science 244: 1288-1292.

Castro, F.O., O. Hernandez, C. Uliver, R. Solano, C. Milanes, A. Aguilar, A. Perez, R. de Armas, L. Herrea, and J. de la Fuente (1991). Introduction of foreign DNA into the spermatozoa of farm animals. Theriogenology 36: 1099-1110.

Cavari, B., B. Funkenstein, T.T. Chen, L.I. Gonzalez-Villasenor, and M. Schartl (1993a). Effect of growth hormone on the growth rate of the gilthead seabream (*Sparus aurata*) and use of different constructs for the production of transgenic fish. Aquaculture 111: 189-197.

Cavari, B., Y. Hong, B. Funkenstein, B. Moav, and M. Schartl (1993b). All-fish gene constructs for growth hormone gene transfer in fish. Fish Physiol. and Biochem. 11: 345-352.

Chabot, B. (1996). Directing alternative splicing: cast and scenarios. Trends in Genetics 12: 472-478.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher (1994). Green fluorescent protein as a marker for gene expression. Science 263: 802-805.

Chatakondi, N., R.T. Lovell, P.L. Duncan, M. Hayat, T.T. Chen, D.A. Powers, J.D. Weete, K. Cummins and R.A. Dunham (1995). Body composition of transgenic common carp, *Cyprinus carpio*, containing rainbow trout growth hormone gene. Aquaculture 138: 99-109.

Chen, H., Y. Yi, M. Chen and X. Yang (1986). Studies on the development potentiality of cultured cell nuclei of fish. Acta Hydrobiol. 10: 1-7.

Chen, T.T., C-M. Lin, R.A. Dunham and D.A. Powers (1992). Integration, expression and inheritance of foreign fish growth hormone gene in transgenic fish. *IN* Hew, C.L. and G.L. Fletcher (eds.) *Transgenic Fish*. World Scientific Pub. Singapore. pp. 2164-175.

Chen, T.T., K. Kight, C.M. Lin, D.A. Powers, M. Hayat, N. Chatakondi, A.C. Ramboux, P.L. Duncan, and R.A. Dunham. (1993). Expression and inheritance of RSVLTR-rtGH1 complementary DNA in the transgenic common carp, *Cyprinus carpio*. Mol. Mar. Biol. Biotech. 2: 88-95.

Chen, T.T., N.H. Vrolijk, J. Lu, C.M. Lin, R. Reimschuessel and R.A. Dunham (1996). Transgenic fish and its application in basic and applied research. Biotechnol. Ann. Rev. 2: 205-235.

Chen, T.T. and D.A. Powers (1990). Transgenic fish. Trends in Biotech. 8: 209-215.

Chong, S.S. C. and Vielkind, J.R. (1989). Expression and fate of CAT reporter gene microinjected into fertilized medaka (*Oryzias latipes)* eggs in the form of plasmid DNA, recombinant phage particles and its DNA. J. Appl. Genet. 78: 369-380.

Chourrout, D., R. Guyomard and L.M. Houdebine. (1986). High efficiency gene transfer in rainbow trout (*Salmo gairdneri Rich.*) by microinjection into egg cytoplasm. Aquaculture 51: 143-150.

Chourrout, D. and E. Perrot (1992). No transgenic rainbow trout produced with sperm incubated with linear DNA. Mol. Mar. Biol. Biotech. 1: 282-285.

Chow, S. (1998). Intron as a source of genetic polymorphism for fish population genetics. *IN* LeGal, Y. and H.O. Halvorson (eds.) *New Developments in Marine Biotechnology*, Plenum Press, N.Y. pp. 231-233.

Christie, K.E. (1997). Immunization with viral antigens: infectious pancreatic necrosis. Dev Biol Stand 90: 191-199.

Cloud, J.G. (1990). Strategies for introducing foreign DNA into the germ line of fish. J. Reprod. Fert. Suppl. 41: 107-116.

Collas, P., and P. Aleström (1997a). Rapid targeting of plasmid DNA to zebrafish embryo nuclei by the nuclear localization signal of SV40 T antigen. Mol. Mar. Biol. Biotech. 6: 48-58.

Collas, P., and P. Aleström (1997b). Nuclear localization signals: a driving force for nuclear transport of plasmid DNA in zebrafish. Biochem. Cell. Biol. 75: 633-640.

Collas, P., H. Husebye and P. Aleström (1996). The nuclear localization sequence of SV40 T antigen promotes transgene uptake and expression in zebrafish embryo nuclei. Transgenic Res. 5: 451-458.

Collas, P., H. Husebye and P. Aleström (1997). Transferring foreign genes into zebrafish eggs by microinjection. *IN* Houdebine, L.M. (ed.) *Transgenic Animals: Generation and Use*. Harwood Academic Pub. Amsterdam. pp. 119-122.

Collodi, P., Y. Kamei, A. Sharps, D. Weber, and D. Barnes. (1992). Fish embryo cell cultures for derivation of stem cells and transgenic chimeras. Mol. Mar. Biol Biotech. 1: 257-265.

Conlon, I. and M. Raff (1999). Size control in animal development. Cell 96: 235-244.

Cook, R.M., A. Sinclair, and G. Stefansson (1997). Potential collapse of North Sea cod stocks. Nature 385: 521-522.

Cormack, B.P., R.H. Valdiva and S. Falkow (1996). FACS-optimized mutants of the green fluorescent protein (GFP). Gene 173: 33-38.

Cui, Z., Z. Zhu, C. Yibo, L. Guohua and X. Kesheng (1996). Food consumption and energy budget in MThGH-transgenic F2 red carp (*Cyprinus carpio* L red var.). Chinese Science Bulletin 41: 591-596.

Cui, Z. and Z. Zhu (1993). Hormonal replacement therapy in fish: human growth hormone gene function in hypophysectomized carp. Fish Physiol. Biochem. 12: 161-169.

Culp, P., C. Nüsslein-Volhard and N. Hopkins (1991). High-frequency germ-line transmission of plasmid DNA sequences injected into fertilized zebrafish eggs. Proc. Natl. Acad. Sci. USA 88: 7953-7957.

Davies, P.L., C.L. Hew and G.L. Fletcher (1988). Fish antifreeze proteins: physiology and evolutionary biology. Can. J. Zool. 66: 2611-2617.

Davies, P.L., G.L. Fletcher and C.L. Hew (1989). Fish antifreeze protein genes and their use in transgenic studies. *IN* Maclean, N. (ed.) *Oxford Surveys on Eukaryotic Genes* 6: 85-109.

de la Fuente, J., R. Martínez, M.P. Estrada, O. Hernández, E. Cabrera, D. Garcia del Barco, R. Lleonart, R. Pimentel, R. Morales, F. Herrera, A. Morales, I. Guillen, and J.C. Piña (1995). Towards growth manipulation in tilapia (*Oreochromos* sp.) generation of transgenic tilapia with chimeric constructs containing the tilapia growth hormone cDNA. J. Mar. Biotech. 3: 216-219.

de la Fuente, J., I. Guillén and M.P. Estrada (1998). The paradox of growth acceleration in fish. *IN* LeGal, Y. and H.O. Halvorson (eds.) *New Developments in Marine Biotechnology*, Plenum Press, N.Y. pp. 7-10.

DePamphilis, M.L., S.A. Herman, E. Martinez-Salas, L.E. Chalifour, D.O. Wirak, D.Y. Cupo and M. Miranda (1988). Microinjecting DNA into mouse ova to study DNA replication and gene expression and to produce transgenic animals. BioTechniques 6: 662-680.

Detrich, H.W., M. Westerfield, and L.I. Zon (eds.) (1999a).*The Zebrafish: Biology* Meth. Cell Biol. vol. 59. Academic Press, San Diego

Detrich, H.W., M. Westerfield, and L.I. Zon (eds.) (1999b).*The Zebrafish: Genetics and Genomics* Meth. Cell Biol. vol. 60. Academic Press, San Diego

Devlin, R.H. (1997). Transgenic salmonids. *IN* Houdebine, L.M. (ed.) *Transgenic Animals.* Harwood Academic Pub. Amsterdam. pp. 105-117.

Devlin, R.H. and E.M. Donaldson (1992). Containment of genetically altered fish with emphasis on salmonids. *IN* Hew, C.L. and G.L. Fletcher (eds.) *Transgenic Fish.* World Scientific Pub. Singapore. pp. 229-265.

Devlin, R.H., T.Y. Yesaki, C. Biagi, E.M. Donaldson, P. Swanson and W.K. Chan (1994a). Extraordinary salmon growth. Nature 371: 209-210.

Devlin, R.H., M. Izadnegahdar, T.Y. Yesaki, E.M. Donaldson and R.M. Shaw (1994b). Craniofacial consequences in transgenic fish expressing growth hormone. J. Dent. Res. 73: 265.

Devlin, R.H., T.Y. Yesaki, E.M. Donaldson, S-J. Du and C.L. Hew (1995a). Production of germline transgenic Pacific salmonids with dramatically increased growth performance. Canad. J. Fish Aquat. Sci. 52: 1376-1384.

Devlin, R.H., T.Y. Yesaki, E.M. Donaldson, and C. Hew (1995b). Transmission and phenotypic effects of an antifreeze/GH construct in coho salmon (*Oncorhynchus kisutch*). Aquaculture 137: 161-167.

de Vos, A.M., M. Ultsch and A.A. Kossiakoff (1992). Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255: 306-312.

Disney, J.E., K.R. Johnson, D.K. Banks and G.H. Thorgaard (1988). Maintenance of foreign gene expression and independent chromosome fragments in adult transgenic rainbow trout and their offspring. J. Exp. Zoo. 248: 335-344.

Dobie, K., M. Mehtali, M. McMlenaghan and R. Lathe (1997) Variegated gene expression in mice. Trends Genet 13: 127-30.

Doefler, W. (1992). DNA methylation: eukaryotic defense against the transcription of foreign genes. Microbial. Pathogenesis 12: 1-8.

Doolittle, W.F. (1997). Fun with genealogy. Proc. Natl. Acad. Sci. USA 94: 12751-12753.

Dorer, D.R. (1997). Do transgene arrays form heterochromatin in vertebrates? Transgenic Res. 6: 3-10.

Down, N.E., E.M. Donaldson, H.M. Dye, T.C. Boone, K.E. Langley, and L.M. Souza (1989). A potent analog of recombinant bovine somatotropin accelerates growth in juvenile coho salmon (*Oncorhynchus kisutch*). Canad. J. Fish Aquat. Sci. 46: 178-183.

Driever, W., L. Solnica-Krezel, A.F. Schier, S.C.F. Neuhaus, J. Malicki, D.L. Stemple, D. Y.R. Stanier, F. Zwartkruis, S. Abdelilah, Z. Rangini, J. Belak and C. Boggs (1996). A genetic screen for mutation affecting embryogenesis in zebrafish. Development 123: 37-46.

Du, S.J., Z. Gong, G.L. Fletcher, M.A. Shears, M.J. King, D.R. Idler and C.L. Hew. (1992a). Growth enhancement in transgenic Atlantic salmon by the use of an "all-fish" chimeric growth hormone gene construct. Bio/Technology 10: 176-181.

Du, S.J., Z. Gong, C.H. Tan, G.L. Fletcher, and C.L. Hew (1992b). Development of an "all fish" gene cassette for gene transfer in aquaculture. Mol. Mar. Biol. Biotech. 1: 290-300.

Dunham, R.A. and R.H. Devlin (1999). Comparison of traditional breeding and transgenesis in farmed fish with implications for growth enhancement and fitness. *IN* Murray, J.D., G.B. Anderson, A.M. Oberbauer and M.M. McGlouoghlin (eds.) *Transgenic Animals in Agriculture*. CABI Publishing, Wallingford, UK. pp. 209-229.

Dunham. R.A. and J. Eash (1987). Transfer of the metallothionein-human growth hormone fusion gene into channel catfish. Trans. Amer, Fish. Soc. 116: 87-91.

Dunham, R.A., A.C. Ramboux, P.L. Duncan, M. Hayat, T.T. Chen, C.M. Lin, K. Kight, L.I. Gonzales-Villasenor, and D.A. Powers (1992). Transfer, expression and inheritance of salmonid growth hormone gene in channel catfish, *Ictalurus punctatus*, and effect on performance traits. Mol. Mar. Biol. Biotech. 1: 380-389.

Eisen, J.S. (1996). Zebrafish make a big splash. Cell 87: 969-977.

Engelking, H.M. and J.C. Leong (1989a). The glycoprotein from infectious hematopoietic necrosis virus elicits neutralizing antibody and protective responses. Virus Res 13: 213-230.

Engelking, H.M. and J.C. Leong (1989b). Glycoprotein from infectious hematopoietic necrosis virus (IHNV) induces protective immunity against five IHNV types. J. Aquat. Anim. Health 1: 291-300.

Essner, J.J., J.G. Laing, E.C. Beyer, R.G. Johnson, and P.B. Hackett (1996). Expression of zebrafish *connexin43.4* in the notochord and tail bud of wild-type and *no tail* mutant embryos. Dev. Biol. 177: 449-462.

Essner, J.J., J.J. Breuer, R.D. Essner, S.C. Fahrenkrug and P.B. Hackett (1997). The zebrafish thyroid hormone receptor $\alpha 1$ is expressed during early embryogenesis and can function in transcriptional repression. Differentiation 62: 107-117.

Essner, J.J., R.G. Johnson and P.B. Hackett (1999). Overexpression of thyroid hormone receptor $\alpha 1$ during zebrafish embryogenesis disrupts hindbrain patterning and implicates retinoic acid receptors in the control of Hox gene expression. Differentiation 65: 1-11.

Evans M.J., M.B.L.Carlton and A.P. Russ (1997) Gene trapping and functional genomics. Trends Genet 13:370-374

Fadool, J.M., D.L. Hartl, and J.E. Dowling (1998). Transposition of the *mariner* element from *Drosophila mauritiana* in zebrafish. Proc. Natl. Acad. Sci. USA 95: 5182-5186.

Fahrenkrug, S.C., K. Clark, and P.B. Hackett (1999). Dicistronic gene expression in developing zebrafish. Mar. Biotech. 1: 552-561.

FAO (1997). Review of the state of world fishery resources: Marine fisheries. FAO Fisheries Circular No. 920 FIRM/C920. Rome
[www.fao.org/WAICENT/FAOINFO/FISHERY/publ/circular/c920/intro]

Fire, A., SQ-Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806-811.

Fishman, M.C. and E.N. Olson (1997). Parsing the Heart: Genetic modules for organ assembly. Cell 91: 153-156.

Fjalestad, K.T., T. Gjdrem, and B. Gjerde (1993). Genetic improvement of disease resistance in fish: an overview. Aquaculture 111: 65-74.

Fletcher, G.L. and P.L. Davies (1991). Transgenic fish for aquaculture. Genetic Engineering 13: 331-370.

Fletcher, G.L., P.L. Davies and C.L. Hew (1992). Genetic engineering of freeze-resistant Atlantic salmon. *In* Hew, C.L. and G.L. Fletcher (eds.) *Transgenic Fish*. World Scientific Publishers, Singapore. pp. 190-208.

Fletcher, G.L., M.A. Shears, J.J. King, P.L. Davies and C.L. Hew (1988). Evidence of antifreeze protein gene transfer in Atlantic salmon (*Salmo salar*). Canad. J. Fish Aquat. Sci. 45: 352-357.

Friedenreich, H. and M. Schartl (1990). Transient expression directed by homologous and heterologous promoter and enhancer sequences in fish cells. Nuc. Acids Res. 18: 3299-3305.

Fu, C., Y. Cui, S.S.O. Hung, and Z. Zhu (1998). Growth and feed utilisation by F4 human growth hormone transgenic carp fed diets with different protein levels. J. Fish Biol. 53: 115-129.

Gaiano, N., M. Allende, A. Amsterdam, K. Kawakami and N. Hopkins (1996a). Highly efficient germ-line transmission of proviral insertions in zebrafish. Proc. Natl. Acad. Sci. USA 93: 7777-7782.

Gaiano, N. and N. Hopkins (1996b). Insertional mutagenesis and rapid cloning of essential genes in zebrafish. Nature 383: 829-832

García-Pozo, S., J. Béjar, M. Shaw and M.C. Alvarez (1998). Effect of exogenous DNA microinjection on early development of the seabream (*Sparus aurata*). Mol. Mar. Biol. Biotech. 7: 248-258.

Garrick, D., S. Fiering, D.I.K. Martin, and E. Whitlaw (1998). Repeat-induced gene silencing in mammals. Nature Genetics 18: 56-59.

Gauvry, L., S. Ennion, E. Hansen, P. Butterworth and G. Goldspink (1996). The characterisation of the 5' regulatory region of a temperature-induced myosin-heavy-chain gene associated with myotomal muscle growth in the carp. Eur. J. Biochem. 236: 887-894.

GenBank (1998). Zebrafish EST sequences available at: www.ncbi.nlm.nih.gov/htbin-post/Taxonomy/wgetorg?name=Danio+rerio&lvl=0.

Geyer, P.K. (1997) The role of insulator elements in defining domains of gene expression. Curr. Opin. Genet. Devel. 7: 242-248.

Gibbs, P.D.L., A. Gray, and G. Thorgaard (1994a). Inheritance of *P* element and reporter gene sequences in zebrafish. Mol. Mar. Biol. Biotech. 3: 317-326.

Gibbs, P.D.L., A. Peek, and G. Thorgaard (1994b). An in vitro screen for luciferase transgene in zebrafish. Mol. Mar. Biol. Biotech. 3: 307-316.

Gill, J.A., J.P. Sumpter, E.M. Donaldson, H.M. Dye, L.M. Souza, T. Berg, J. Wypych, and K.E. Langley (1985). Recombinant chicken and bovine growth hormone accelerate growth in aquacultured juvenile Pacific salmon (*Oncorhynchus kisutch*). Bio/Technology 3: 643-646.

Gómez-Chiarri, M., S.K. Livingston, C. Muro-Cacho, S. Saunders and R.P. Levine (1996a). Introduction of foreign genes into the tissue of live fish by direct injection and particle bombardment. Dis. Aquat. Org. 27: 5-12.

Gómez-Chiarri, M, L.L. Brown and R.P. Levine (1996b). Protection against *Renibacterium salmoninarum* infection by DNA-based immunization. Aquaculture Biotechnology Symposium Proceedings. Physiol. Sect. Amer. Fish. Soc.155-157.

Gómez-Chiarri, M. and L.A. Chiaverini (1999). Evaluation of eukaryote promoters for the construction of DNA vaccines for aquaculture. (submitted).

Gong, Z. and C.L. Hew (1995). Transgenic fish in aquaculture and developmental biology. Curr. Top. Dev. Biol. 30: 177-214.

Gong, Z., Z. Hu, Q. gong, R. Kitching and C.L. Hew (1995). Bulk isolation and identification of fish genes by cDNA clone tagging. Mol. Mar. Biol. Biotech. 3: 243-251.

Gong, Z., C.L. Hew and J.R. Vielkind (1991). Functional analysis and temporal expression of promoter regions from fish antifreeze protein genes in transgenic Japanese medaka embryos. Mol. Mar. Biol. Biotech. 1: 64-72.

Gong, Z., T. Yan, J. Liao, S.E. Lee, J. He, and C.L. Hew (1997). Rapid identification and isolation of zebrafish clones. Gene 201: 87-98.

Gordon, J.W. and F.H. Ruddle (1981). Integration and stable germ-line transmission of genes injected into mouse pronuclei. Science 214: 1244-1246.

Gross, M.L., J.F. Schneider, N. Moav, B. Moav, M.C. Alvarez, S.H. Myster, Z. Liu, E.M. Hallerman, P.B. Hackett, K.S. Guise, A.J. Faras and A.R. Kapuscinski (1992). Molecular analysis and growth evaluation of northern pike (*Esox lucius*) microinjected with growth hormone genes. Aquaculture 103: 253-273.

Grobet, l., D. Poncelet, L.J. Royo, B. Brpiwers. D. Pirottin,l C. Michaux, L. Menissier, M. Zanotti, S. Dunner, and M. Georges (1998). Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. Mammal. Genome 9: 210-213.

Guillén, I., J. Berlanga, C.M. Valenzuela, A. Morales, J. Toledo, M.P. Estrada, P. Puentes, O. Hayes, and J. de la Fuente (1999). Safety evaluation of transgenic tilapia with accelerated growth. Mar. Biotech. 1: 2-14.

Guise, K.S., P.B. Hackett and A.J. Faras (1992). Transfer of genes encoding neomycin resistance, chloramphenicol acetyl transferase, and growth hormone into goldfish and northern pike. *IN* C. Hew and G.L. Fletcher (eds.). *Transgenic Fish.* World Scientific Publishing Co. Singapore. 142-163.

Guise, K.S., A.R. Kapuscinski, P.B. Hackett and A.J. Faras (1991). Gene transfer in fish. *IN* N.L. First and F.P. Haseltine (eds.). *Transgenic Animals.* Butterworth-Heinemann, Boston, MA 295-306.

Gurdon, J. B. (1986). Nuclear transplantation in eggs and oocytes. J Cell Sci (Suppl.) 4: 287-318.

Guyomard, R., D. Chourrout, C. Leroux, L.M. Houdebine and F. Pourrain (1989). Integration and germ line transmission of foreign genes microinjected into fertilized trout eggs. Biochimie 71: 857-863.

Hackett, P.B. (1993). The molecular biology of transgenic fish. *IN Biochemistry and Molecular Biology of Fishes* Hochachka, P.W. and T.M. Mommsen (eds.) Elsevier, Amsterdam. Vol. 2, pp. 207-240.

Hackett, P.B., Zs. Izsvák, Z. Ivics, and L. Caldovic (1999). Development of genetic tools for transgenic animals. *IN* Murray, J.D., G.B. Anderson, A.M. Oberbauer and M.M. McGlouoghlin (eds.) *Transgenic Animals in Agriculture.* CABI Publishing, Wallingford, UK. pp. 19-35.

Haffter, P., M. Granato, M. Brand, M.C. Mullins, M. Hammerschmidt, D.A. Kane, J. Odenthal, F.J.M. van Eeden, Y-J. Jiang, C-P. Heisenberg, R.N. Kelsh, M. Furutani-Seiki, E. Vogelsang, D. Beuchle, U. Schach, C. Fabian and C. Nüsslein-Volhard (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. Development 123: 1-36.

Hallerman, E.M., and A.R. Kapuscinski (1992). Ecological and regulatory uncertainties associated with transgenic fish. *IN* Hew, C.L. and G.L. Fletcher (eds.) *Transgenic Fish.* World Scientific Publishers, Singapore. pp. 209-228.

Hallerman, E.M., J.F. Schneider, M. Gross, A.J. Faras, P.B. Hackett, K.S. Guise, and A.R. Kapuscinski (1988). Enzymatic dechorionation of goldfish, walleye, and northern pike eggs. Trans. Am. Fish. Soc. 117: 456-460.

Hallerman, E.M., J.F. Schneider, M. Gross, Z. Liu, S.J. Yoon, L. He, P.B. Hackett, A.J. Faras, A.R. Kapuscinski, and K.S. Guise (1990). Gene expression promoted by the RSV long terminal repeat lelement in transgenic goldfish. Animal Biotech. 1: 79-93.

Hamada, K., K. Tamaki, T. Sasado, Y. Watai, S. Kani, Y. Wakamatsu, K. Ozato, M. Kinoshitqa, R. Kohno, S. Takagi and M. Kimura (1998). Usefulness of the medaka β-actin promoter investigation using a mutant GFP reporter gene in transgenic medaka (*Oryzias latipes*). Mol. Mar. Biol. Biotech. 7: 173-180.

Hammer, R.E., V.G. Pursel, C.E. Rexroad Jr., R.J. Wall, D.J. Bolt, K.M. Ebert, R.D. Palmiter and R.L. Brinster (1985). Production of transgenic rabbits, sheep, and pigs by microinjection. Nature 315: 680-683.

Hansen, E., K. Fernandes, G. Goldspink, P. Butterworth P.K. Umeda, and K.C. Chang (1991). Strong expression of foreign genes following direct injection into fish muscle. FEBS Lett. 290: 73-76.

Harrington. J.J., G.V. Bokkelen, R.W. Mays, K. Gustashaw and H.F. Willard (1997). Formation of de novo centromeres and construction of first-generation human artificial microchromosomes. Nature Genetics 15: 345-355.

Hart, D., G.N. Frerichs, A. Rambaut, and D.E. Onions (1996). Complete nucleotide sequence and transcriptional analysis of the snakehead fish retrovirus. J. Virol. 70: 3606-3616.

Haseloff, J. and W.L. Gerlach (1988). Simple RNA enzymes with new and highly specific endoribonuclease activities. Nature 334: 585-591.

Hastie, N.D. and J.O. Bishop (1976). The expression of three abundance classes of messenger RNA in mouse tissues. Cell 9: 761-774.

Henikoff, S. (1998) Conspiracy of silence among repeated transgenes. BioEssays 20: 532-534.

Heppell J, T. Wu, N. Lorenzen, A.E. Ellis, S.E., Efler, N.K. Armstrong, J Schorr and H.L. Davis (1998). Development of DNA vaccines for aquaculture. *IN*: LeGal, Y. and H.O. Halvorson (eds.) *New Developments in Marine Biotechnology*, Plenum Press, N.Y. pp. 97-100.

Hernández, O., F.O. Castro, A. Aguilar, A. Uliyer, A. Péréz, L. Herrera, and J. de la Fuente (1991). Gene transfer in common carp (*Cyprinus carpio* L.) by microinjection into the germinal disc. Theriogenology 35: 625-632.

Hernández, O, I. Guillen, M.P. Estrada, E. Cabrera, R. Pimentel, J.C. Pina, Z. Abad, V. Sanchez, Y. Hidalgo, R. Martinez, R. Lleonart and J. de la Fuente (1997) Characterization of

transgenic tilapia lines with different ectopic expression of tilapia growth hormone. Mol. Mar. Biol. Biotechnol. 6: 364-75.

Herschberger, W.K., J.M. Meyers, R.N. Iwamoto, W.C. McAuley, and A.M. Saxon (1990). Genetic changes in the growth of coho salmon (*Oncorhynchus kisutch*) in marine net-pens produced by ten years of selection. Aquaculture 85: 187-198.

Heyner, S. and W.T. Garside (1994). Biological actions of IGFs in mammalian development. BioEssays 16: 55-57.

Hew, C. (1989). Transgenic fish: present status and future directions. Fish Physiol. Biochem. 7: 409-413.

Hew, C.L., N.-C. Wang, S. Joshi, G.L. Fletcher,, G.K. Scott, P.H. Hayes, B. Buettner, and P.L. Davies (1988). Multiple genes provide the basis for antifreeze protein diversity and dosage in the ocean pout, *Macrozoarces americanus*. J. Biol. Chem. 263: 12049-12055.

Hew, C.L., P.L. Davies and G. Fletcher (1992). Antifreeze protein gene transfer in Atlantic salmon. Mol. Mar. Biol. Biotech. 1: 309-317.

Higashijima, S., H. Okamoto, N. Ueno, Y. Hotta and G. Eguchi (1997). High-frequency generation of transgenic zebrafish which reliably express GFP in whole muscles or the whole body by using promoters of zebrafish origin. Dev Biol 192: 289-299.

Holzschu, D.L., D. Martineau, S.K. Fodor, V.M. Vogt, P. Bowser, and J.W. Casey (1995). Nucleotide sequence and protein analysis of a complex retrovirus, walleye dermal sarcoma virus. J. Virol. 69: 5320-5331.

Hong, Y., C. Winkler, G. Brem and M. Schartl (1993). Development of a heavy metal-inducible fish-specific expression vector for gene transfer in vitro and in vivo. Aquaculture 111: 215-226.

Hong, Y. and M. Schartl (1996a). Establishment and growth responses of early medakafish (*Oryzias latipes*) embryonic cells in feeder layer-free cultures. Mol. Mar. Biol. Biotech. 5: 93-104.

Hong, Y., C. Winkler and M. Schartl (1996b). Pluripotency and differentiation of embryonic stem cell lines from the medakafish. Mech. Dev. 60: 33-44.

Hong, Y. and M. Schartl (1996c). Embryonic stem cells, cell transplantation and nuclear transplantation in fish. *IN* Haffray, P. (ed.) *Preservation techniques applied to diversity management in fish farming*. Bordeaux Aquaculture, Bordeaux, France. pp 475-488.

Hong, Y., C. Winkler, and M. Schartl (1998). Production of medakafish chimeras from a stable embryonic stem cell line. Proc. Natl. Acad. Sci. USA 95: 3679-3684.

Houdebine, L.M. and D. Chourrout (1991). Transgenesis in fish. Experimentia 47: 891-897.

Hsieh, C.L. (1997). Stability of patch methylation and its impact in regions of transcriptional initiation and elongation. Mol. Cell. Biol. 17: 5897-5904.

Huang, M.T.F. and C.M. Gorman (1990a). Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. Nuc. acids Res. 18: 939-947.

Huang, M.T.F. and C.M. Gorman (1990b). The simian virus 40 small-t intron, present in many common expression vectors, leads to aberrant splicing. Mol. Cell. Biol. 10: 1805-1810.

Huelseweh, B., B. Kohl, H. Hentschel, R.K. Kinne and A. Werner (1998). Translated anti-sense product of the Na/phosphate co-transporter (NaPi-II). Biochem. J. 332: 483-489.

Hyatt, T.M. and S.C. Ekker (1999). Vectors and techniques for ectopic gene expression in zebrafish. Meth. Cell Biol. 59:117-126

Hyodo-Takuchi, Y, C. Winkler, Y. Kurihara, A. Schartl and M. Schartl (1997). Phenotypic rescue of the albino mutation in the medakafish (*Oryzias latipes*) by a mouse tyrosinase transgene. Mech. Dev. 68: 27-35.

Hyrien, O., Ch. Maric, and M. Mechali (1995). Transition in specification of embryonic metazoan DNA replication origins. Science 70: 994-997.

Inagaki, H, Y. Bessho, A. Koga, and H. Hori (1994). Expression of the tyrosinase-encoding gene in a colorless melanophore mutant of the medaka fish (*Oryzias latipes*). Gene 150: 319-324.

Indig, F.E. and B. Moav (1992). A prokaryotic gene is expressed in fish cells and persists in tilapia embryos following microinjection through the micropyle. *IN* Zohar, Y and B. Breton (eds.) *Reproduction in Fish--Basic and Applied Aspects in Endocrinology and Genetics.* INRA Press, Paris 138-141.

Inoue, K. (1992). Expression of reporter genes introduced by microinjection and electroporation in fish embryos and fry. Mol. Mar. Biol. Biotech. 1: 266-270

Inoue, K., N. Akita, T. Shiba, M. Satake, and S. Yamashita (1992a). Metal-inducible activities of metallothionein promoter. Biochim. Biophys. Res. Comm. 185: 1108-1114.

Inoue, K., S. Asada, S. Kabeno, S. Yamashita, E. Nagahisa and M. Satake (1992b). Specific stages of oocyte maturity in medaka to obtain efficient foreign gene transfer by microinjection. Nippon Suisan Gakkaishi 58: 1251-1255.

Inoue, K., K. Ozato, H. Kondoh, T. Iwamatsu, Y. Wakamatsu, T. Fujita and T.S. Okada (1989). Stage-dependent expression of the chicken δ-crystallin gene in transgenic fish embryos. Cell Diff. Dev. 27: 57-68.

Inoue, K., S. Yamashita, J. Hata, S. Kabeno, S. Asada, E. Nagahisa and T. Fujita (1990). Electroporation as a new technique for producing transgenic fish. Cell Differ. Dev. 29: 123-128.

Ivics, Z, Zs. Izsvák, and P.B. Hackett (1993). Enhanced incorporation of transgenic DNA into zebrafish chromosomes by a retroviral integration protein. Mol. Mar. Biol. Biotech. 2: 162-173.

Ivics, Z., Zs. Izsvák, A. Minter, and P.B. Hackett (1996). Identification of functional domains and evolution of Tc1 family of transposable elements. Proc. Natl. Acad. Sci. USA 93: 5008-5013.

Ivics, Z., Zs. Izsvák, and P.B. Hackett (1997). Molecular reconstruction of *Sleeping Beauty*, A *Tc1*-likew transposon from fish, and its transposition in human cells. Cell 91: 501-510.

Ivics, Z., Z, Izsvák and P.B. Hackett (1999). Genetic applications of transposons and other repetitive elements in zebrafish. *IN* Detrich, H.W., M. Westerfield, and L.I. Zon (eds.) *The Zebrafish: Genetics and Genomics* Meth. Cell Biol. 60: 99-131.

Iyengar, A. and N. Maclean (1995). Transgene concatemerization and expression in rainbow trout (*Oncorhynchus mykiss*). Mol. Mar. Biol. Biotech. 4: 248-254.

Iyengar, A., F. Müller, and N. Maclean (1996). Expression and regulation of transgenes in fish. Transgenic Res. 5: 147-165.

Izant, J.G. and H. Weintraub (1985). Constitutive and conditional suppression of exogenous and endogenous genes by anti-sense RNA. Science 229: 345-351.

Izsvák, Zs., Z. Ivics and P.B. Hackett (1995). Characterization of a Tc1-like transposable element in zebrafish (*Danio rerio*). Mol. Gen. Genet. 247:312-322.

Izsvák, Zs., Z. Ivics, D. Garcia-Estefania, S.C. Fahrenkrug, and P.B. Hackett (1996). DANA elements: a family of composite, short interspersed, DNA elements associated with mutational activities in zebrafish (*Danio rerio*). Proc. Natl. Acad. Sci. USA 93: 1044-1048.

Izsvák, Zs., Z. Ivics, and P.B. Hackett (1997). Repetitive elements and their genetic applications in zebrafish. Biochem. Cell Biol. 75: 507-523.

Izsvák, Zs., Z. Ivics, N. Shimoda, D. Mohn, H. Okamoto and P.B. Hackett (1999). Short inverted repeat transposable elements in teleost fish and implications for their mechanism of amplification. J. Mol. Evol. 48: 13-21.

Jackson, R.J. and A. Kaminski (1995). Internal initiation of translation in eukaryotes: the picornavirus paradigm and beyond. RNA 1:985-1000

Jain, R., M.C. Rivera and J.A. Lake (1999). Horizontal gene transfer among genomes: The complexity hypothesis. Proc. Natl. Acad. Sci. USA 96: 3801-3806.

Jiang, Y. (1993). Transgenic fish -- gene transfer to increase disease and cold resistance. Aquaculture 111: 31-40.

Jones, P.A. (1999). The DNA methylation paradox. Trends Genet. 15: 34-37.

Kane, D.A. and C.B. Kimmel (1993). The zebrafish midblastula transition. Development 119: 447-456.

Kapuscinski, A.R. and E.M. Hallerman (1991). Implications of introduction of transgenic fish into natural ecosystems. Canad. J. Fisheries and Aquatic 48: 99-107.

Kapuscinski, A.R. and E.M. Hallerman (1990). Transgenic Fishes. Fisheries 15: 2-5.

Kass, S.U., J.P. Goddard, and R.L.P. Adams (1993). Inactive chromatin spreads from a focus of methylation. Mol. Cell. Biol. 13: 7372-7379.

Kawakami, K., A. Koga, H. Hori, and A. Shima (1998) Excision of the *Tol2* transposable element of the madaka fish, *Oryzias latipes*, in zebrafish, *Danio rerio*. Gene 225: 17-22.

Kaufman, C.D., G. Martínez-Rodriguez and P. B. Hackett, (1999) Ectopic expression of c-*ski* disrupts gastrulation and neural patterning in zebrafish. Mech. Dev. (submitted)

Kermekchiev, M., M. Pettersson, P. Matthias and W. Schaffner (1991). Every enhancer works with every promoter for all the combinations tested: could new regulatory pathways evolve by enhancer shuffling? Gene Expression 1: 71-81.

Kellum, R. and P. Schedl (1991) A position-effect assay for boundaries of higher order chromosomal domains. Cell 64: 941-950.

Kennerdell, J.R. and R.W. Carthew (1998). Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. Cell 95: 1017-1026.

Khoo, H-W., L-H. Ang, H-B. Lim and K-Y Wong (1992). Sperm cells as vectors for introducing foreign DNA into zebrafish. Aquaculture 107: 1-19.

Kinoshita, M., H. Toyhara, M. Sakaguchi, K. Inoue, S. Yamashita, M. Satake, Y. Wakamatsu, and K. Ozato (1994). A stable line of transgenic medaka (*Oryzias latipes*) carrying the CAT gene. Aquaculture 143: 267-276 .

Kinoshita, M., H. Toyhara, M. Sakaguchi, N. Kioka, T. Komano, K. Inoue, S. Yamashita, M. Satake, Y. Wakamatsu, and K. Ozato (1996) Zinc-induced activation of rainbow trout metallothionine-A promoter in transgenic medaka. Fisheries Sci. 60: 307-309.

Klein, T.M., E.D. Wolf, R. Wu and J.C. Sanford (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70-73.

Knibb, W. (1996). Risk form genetically engineered and modified marine fish. Trans. Res. 5: 1-9.

Koener, J.F. and J.C. Leong (1990). Expression of the glycoprotein gene from a fish rhabodovirus by using baculovirus vectors. J. Virol. 64: 428-430.

Koga, A. and H. Hori (1997). Albinism due to transposable element insertion in fish. Pigment Cell Res. 10: 377-381.

Köster, R. R. Götz, J. Altschmied, M. Sendtner, and M. Schartl (1996). Comparison of monocistronic and bicistronic constructs for neutrophin transgene and reporter gene expression in fish cells. Mol. Mar. Biol. Biotech. 5: 1-8.

Kotani, H. and E.B. Kmiec (1994a). Transcription activates RecA-promoted homologous pairing of nucleosomal DNA. Mol. Cell. Biol. 14: 1949-1955.

Kotani, H. and E.B. Kmiec (1994b). A role for RNA synthesis in homologous pairing events. Mol. Cell. Biol. 14: 6097-6106.

Kramer, A. (1996). The structure and function of proteins involved in mammalian pre-mRNA splicing. Ann. Rev. Biochem. 65: 367-409.

Kren, B.T., P. Bandyopadhyay and C.J. Steer (1998). *In vivo* site-directed mutagenesis of the *factor IX* gene by chimeric RNA/DNA oligonucleotides. Nature Medicine 4: 285-290.

Kren, B.T., A. Cole-Strauss, E.B. Kmiec and C.J. Steer (1997). Targeted nucleotide exchange in the alkaline phosphatase gene of HuH-7 cells mediated by a chimeric RNA/DNA oligonucleotide. Hepatology 25: 1462-1468.

Krebs, J.E. and M. Dunaway (1998) The scs and scs' insulator elements impart a cis requirement on enhancer-promoter interactions. Mol Cell 1: 301-308.

Kringstein, A.M., F.M.V. Rosi, A. Hoffman, and H.M. Blau (1998). Graded transcriptional response to different concentrations of a single transactivator. Proc. Natl. Acad. Sci. USA 95: 13670-13675.

Lampe, D.J., M.E. Churchill and H.M. Robertson (1996). A purified mariner transposase is sufficient to mediate transposition *in vitro*. EMBO J. 15: 5470-5479.

Lavitrano, M., A. Camaioni, V.M. Fazio, S. Dolci, M.G. Farace, and C. Spadafora (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation in mice. Cell 57:717-723.

Leff, S.E., M.G. Rosenfeld and R.M. Evans (1986). Complex transcriptional units: diversity in gene expression by alternative splicing. Ann. Rev. Biochem. 55: 1091-1117.

Leong, J.C., E. Anderson, L.M. Bootland, OP.W. Chiou, M. Johnson, C. Kim, D. Mourich and G. Trobridge (1997). Fish vaccine antigens produced or delivered by recombinant DNA technologies. Dev. Biol. Stand. 90: 267-277.

Li, M. and R.T. Lovell (1992). Comparison of satiate feeding and restricted feelding of channel catfish with various concentrations of dietary protein in production ponds. Aquaculture 103: 165-175.

Lillehaug, A. (1997). Vaccination strategies in seawater cage culture of salmonids. Dev Biol Stand 90: 401-408.

Lin, S., W. Long, J. Chen, and N. Hopkins (1992). Production of germ-line chimeras in zebrafish by cell transplants from genetically pigmented to albino embryos. Proc. Natl. Acad. Sci. USA 89: 4519-4523.

Lin, S., N. Gaiano, P. Culp, J.C. Burns, T. Friedman, J.K. Yee and N. Hopkins (1994a). Integration and germ-line transmission of a pseudotyped retroviral vector in zebrafish. Science 265: 666-669.

Lin, S., S. Yang and N. Hopkins (1994b). *lacZ* expression in germline transgenic zebrafish can be detected in living embryos. Dev. Biol. 161: 77-83.

Lira, S., E.B. Crenshaw, III, C.K. Glass, L.W. Swanson and M.G. Rosenfeld (1988). Identification of rat growth hormone genomic sequences targeting pituitary expression in transgenic mice. Proc. Natl. Acad. Sci. USA 85: 4755-4759.

Liu, Z., Z. Zhu, K. Roberg, A. Faras, K. Guise, A.R. Kapuscinski, and P.B. Hackett (1990a). Isolation and characterization of the ß-actin gene of carp (*Cyprinus carpio*). DNA Sequence 1: 125-136.

Liu, Z., B. Moav, A.J. Faras, K.S. Guise, A.R. Kapuscinski and P.B. Hackett (1990b). Functional analysis of elements affecting expression of the ß-actin gene of carp. Mol. Cell. Biol. 10: 3432-3440.

Liu, Z., B. Moav, A.J. Faras, K.S. Guise, A.R. Kapuscinski and P.B. Hackett (1990c). Importance of the *CArG* box in regulation of ß-actin-encoding genes. Gene 108: 211-217.

Liu, Z., B. Moav, A.J. Faras, K.S. Guise, A.R. Kapuscinski, and P.B. Hackett (1990d). Development of expression vectors for gene transfer into fish. Bio/Technology 8: 1268-1272.

Liu, Z. and R.A. Dunham (1998). Genetic linkage and QTL mapping of Ictalurid catfish. Circular 321 Alabama Agricultural Experiment Station. Auburn, AL. 1-19.

Liu, Z., P. Li, J. Argu and R.A. Dunham (1998a). Inheritance of RAPD markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. furcatus*), and their F1, F2 and backcross hybrids. Anim. Genet. 29: 58-62.

Liu, Z., A. Nichols, P. Li, and R.A. Dunham (1998b). Inheritance and usefulness of AFLP markers in channel catfish (*Ictalurus punctatus*), blue catfish (*I. Furcatus*), and their F1, F2 and backcross hybrids. Mol. Gen. Genet. 258: 260-268.

Liu, Z., Li, P., Kucuktas, H., Nichols, A., Tan, G., Zheng, X., Argue, B., Dunham, R.A., and D.R. Yant (1999). Development of amplified fragment length polymorphism (AFLP) markers suitable for genetic linkage mapping of catfish. Trans. Amer. Fish. Soc. 128: 317-327.

Long, Q., A. Meng, H. Wang, J.R. Jessen, M.J. Farrell and S. Lin (1997). GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. Development 124: 4105-4111.

Longo L, A. Bygrave, F.G. Grosveld and P.P Pandolfi (1997). The chromosome make-up of mouse embryonic stem cells is predictive of somatic and germ cell chimaerism. Trans. Res. 6:321-328.

Lowary, P.T. and J. Widom (1997). Nucleosome packaging and nucleosome positioning of genomic DNA. Proc. Natl. Acad. Sci. USA 94: 1183-1188.

Lu, J.K., T.T. Chen, C.L. Chrisman, O.M. Andrisani, and J.E. Dixon (1992). Integration, expression and germ-line transmission of foreign growth hormone genes in medaka (*Oryzias latipes*). Mol. Mar. Biol. Biotech. 1: 366-375.

Lyons, G.E., B.K. Micales, M.J. Herr, S.K. Horrigan, S. Namciu, D. Shardy and E. Stavnezer (1994). Protooncogene c-ski is expressed in both proliferating and postmitotic neuronal populations. Dev. Dyn. 201: 354-365.

Maclean, N. (1988). Regulation and exploitation of transgenes in fish. Mut. Res. 399: 255-266.

Maclean. N., A. Iyengar, A. Rahman, Z. Sulaiman and D. Penman (1992). Transgene transmission and expression in rainbow trout and tilapia. Introduction of novel genes into fish. Mol. Mar. Biol. Biotech. 1: 355-365.

Maclean. N., D. Penman and Z. Zhu (1987). Introduction of novel genes into fish. *Bio/Techology* 5: 257-261.

Maclean. N. and A. Rahman (1994). Transgenic fish. *IN* Maclean, N. (ed.) *Animals with novel genes*. Cambridge University Press, Cambridge pp. 63-105.

Malakoff, D. (1997). Extinction on the high seas. Science 277: 486-488.

Mansour, S.L., K.R. Thomas and M.R. Capecchi (1988). Disruption of the protoncogene *int-2* in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336: 348-352.

Martienssen, R. (1998). Transposons, DNA methylation and gene control. Trends Genet. 14: 263-264.

Martin, C.C. and R. McGowan (1995). Genotype specific modifiers of transgene methylation and expression in zebrafish, *Danio rerio*. Genet. Res. Camb. 65: 21028.

Martin, C.C., L. Laforest, M-A. Akimenko and M. Ekker (1999). A role for DNA methylation in gastrulation and somite patterning. Dev. Biol. (in press).

Martineau, D., P.R. Bowser, R.R. Renshaw, and J.W. Casey (1992). Molecular characterization of a unique retrovirus associated with a fish tumor. J. Virol. 66: 596-599.

Martínez, R., M.P. Estrada, J. Berlange, I. Guillén, O. Hernández, E. Cabrera, R. Pimentel, R. Morales, F. Herrera, A. Morales, J.C. Piña, Z. Abad, V. Sánchez, P. Melamed, R. Lleonart, and J. de la Fuente (1996). Growth enhancement in transgenic tilapia by ectopic expression of tilapia growth hormone. Mol. Mar. Biol. Biotech. 5: 62-70.

Masood, E. (1997a). Fisheries science: all at sea when it comes to politics? Nature 386: 105-106.

Masood, E. (1997b). Fishing by numbers reveals its limits. Nature 386: 110.

Masood, E. (1997c). Aquaculture: a solution or source of new problems? Nature 386: 109.

Matsumoto, J. T. Akiyama, E. Hirose, M. Nakamura, H. Yamamoto, and T. Takeuchi (1992). Expression and transmission of wild-type pigmentation in the skin of transgenic orange-colored variants of medaka (*Oryzias latipes*) bearing the gene for mouse tyrosinase. Pigment Cell Res. 5: 322-327.

McCall, M.J., P. Hendry and J.A. Jennings (1992). Minimal sequence requirements for ribozyme activity. Proc. Natl. Acad. Sci. USA 89: 5710-5714.

McEvoy, T.G., F. Gannon and J.M. Sreenan (1992). Gene transfer in fish: potential and practice. Anim. Biotech. 3: 221-243.

McEvoy, T.G., M. Stack, T. Barry, B. Keane, F. Gannon and J.M. Sreenan (1987). Direct gene transfer by microinjection. Theriogenology 27: 258.

McEvoy, T.G., M. Stack, B. Keane, T. Barry, J. Sreenan and F. Gannon (1988). The expression of a foreign gene in salmon embryos. Aquaculture 68: 27-37.

Mc Grath, J. and D. Solter (1983). Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 220, 1300-1302.

McKnight, R.A., A. Shamay, L. Sankaran, R.J. Wall and L. Hennighausen (1992) Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. Proc. Natl. Acad. Sci. USA 89: 6943-6947.

McKnight, S.L. (1997). Gatekeepers of organ growth. Proc. Natl. Acad. Sci. USA 94: 12249-12250.

McPherron, A.C. and S.J. Lee (1997). Double-muscling in cattle due to mutations in the myostatin gene. Proc. Natl. Acad. Sci. USA 94: 12457-12461.

McPherron, A.C., A.M. Lawler and S.J. Lee (1997). Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. Nature 387: 83-90.

Mench, J.A. (1999). Ethics, animal welfare and transgenic farm animals. *IN* Murray, J.D., G.B. Anderson, A.M. Oberbauer and M.M. McGloughlin (eds.) *Transgenic Animals in Agriculture*. CABI Publishing, Wallingford, UK. pp. 251-268.

Meng, A., H. Tang, B.A. Ong, M.J. Farrell and S. Lin (1997). Promoter analysis in living zebrafish embryos indentifies a cis-acting motif required for neuronal expresion of GATA-2. Proc. Natl. Acad. Sci. USA 94: 6267-6272.

Meng, A., J.R. Jessen and S. Lin (1999). Transgenesis. *IN* Detrich, H.W., M. Westerfield, and L.I. Zon (eds.) *The Zebrafish: Genetics and Genomics*. Meth. Cell Biol. 60: 133-148.

Mialhe, E., E. Bachere, V. Buolo, J.P. Cadoret, C. Rousseau, C. Cedeño, E. Saraiva, L. Carrera, J. Calderon, and R.R. Colwell (1995). Future of biotechnology based control of disease in marine invertebrates. Mol. Mar. Biol. Biotech. 4: 275-283.

Moav, B., Z. Liu, Y. Groll and P.B. Hackett (1992a). Selection of promoters for gene transfer into fish. Mol. Mar. Biol. Biotech. 1: 338-345.

Moav, B., Z. Liu, N.L. Moav, L.D. Caldovic, M.L. Gross, A.R. Kapuscinski, A.J. Faras, K.S. Guise and P.B. Hackett (1992b). Expression of heterologous genes in transgenic fish. *IN* Hew, C.L. and G.L. Fletcher (eds.) *Transgenic Fish*. World Scientific Publishers, Singapore. pp. 120-141.

Moav, B., Z. Liu, N.L. Moav, L. Caldovic, M.L. Gross, A.J. Faras, and P.B. Hackett (1993). Regulation of expression of transgenes in developing fish. Trans. Res. 2: 153-161.

Moav, B. (1994). Developmental and molecular genetics in fish. Israel J. Zool. 40: 441-466.

Moffat, A.S. (1998). Improving gene transfer into livestock. Science 282: 1619-1620.

Montgomery, M.K. and AS. Fire (1998). Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. Trends in Genetic. 14: 255-258.

Mulcahy, D. and R.J. Pascho (1984). Adsorption to fish sperm of vertically transmitted fish viruses. Science 325: 33-335.

Müller, F., Z. Ivics, F. Erdélyi, T. Papp, L. Váradi, L. Horváth, N. Maclean and L. Orban (1992). Introducing foreign genes into fish eggs by electroporated sperm as a carrier. Mol. Mar. Biol. Biotech. 1: 276-281.

Müller, F., Z. Lele, L. Váradi, L. Menczel and L. Orbán (1993). Efficient transient expression system based on square pulse electroporation and in vivo luciferase assay of fertilized fish eggs. FEBS Lett. 324: 27-32.

Müller, F., D.W. Williams, J. Kobolak, L, Gauvry, G. Goldspink, L. Orbán and N. Maclean (1997). Activator effect of coinjected enhancers on the muscle-specific expression of promoters in zebrafish embryos. Mol. Reprod. Dev. 47: 404-412.

Müller, H.-P., J.M. Sogo and W. Schaffner (1989). An enhancer stimulates transcription in trans when attached to the promoter via a protein bridge. Cell 58: 767-777.

Murakami, Y, K. Motoshashi, K. Yano, K. Ikebukuro, K. Yokoyama, E. Tamiya and I. Karube (1994). Micromachined electroporation system for transgenic fish. J. Biotechnol. 34: 35-42.

Nam, Y.K., C.H. Jeong and D.S. Kim (1998a). A simple clearing method for improved visualization of *lacZ* expression in fish larvae. Transgenic Res. 7: 397-399.

Nam, Y.K., C.H. Noh and D.S. Kim (1998b). Transmission and expression of an integrated reporter construct in three generations of transgenic mud loach. Aquaculture 172: 229-245.

Namciu, S., Lyons, G.E., Micales, B.K., Heyman, H.C., Colmenares, C., and Stavnezer, E. (1995). Enhanced expression of mouse c-*ski* accompanies terminal skeletal muscle differentiation in vivo and in vitro. Dev. Dyn. 204: 291-300.

Naylor, R.L., R.J. Goldburg, H. Mooney, M. Beveridge, J. Clay, C. Folke, N. Kautsky, J. Lubchenco, J. Primavera, M. Williams (1998). Natures subsidies to shrimp and salmon farming. Science 282: 883-884.

Nelson, D.L., S.A. Ledbetter, L. Corbo, M.F. Victoria, R. Ramirez-Solis, T.D. Webster, D.H. Ledbetter, and C.T. Caskey (1989). Alu polymerase chain reaction: a method for rapid isolation of human-specific sequences from complex DNA sources. Proc. Natl., Acad. Sci. USA 86: 6686-6690.

Neugebauer, K.M. and M.B. Roth (1997). Transcription units as RNA processing units. Genes Dev. 11: 3279-3285.

Neumann, E., M. Schaefer-Ridder, Y. Wang and P.H. Hofschneider (1982). Gene transfer into mouse myloma cells by electroporation in high electric fields. EMBO J. 1: 841-845.

Newport, J. (1987). Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. Cell 48: 205-217.

Newport, J. and M. Kirschner (1982a) A major developmental transition in early Xenopus embryos: I. Characterization and timing of cellular changes at the midblastula stage. Cell 30: 675-686.

Newport, J. and M. Kirschner (1982b) A major developmental transition in early Xenopus embryos: I. Control of the onset of transcription. Cell 30: 687-696.

Nilsson, E. and J.G. Cloud (1992). Rainbow trout chimeras produced by injection of blastomeres into recipient blastulae. Dev. Biol. 89: 9425-9428.

Nomura, T., M.M. Khan, S.C. Kaul, H-D. Dong, R. Wadhwa, C. Colmenares, I. Kohno and S. Ishii (1999). Ski is a component of the histone deacetylase complex required for transcriptional repression by Mad and thyroid hormone receptor. Genes Dev. 13: 412-423.

O'Connell, M. and J.M. Wright (1997). Microsatellite DNA in fishes. Rev. Fish. Biol. Fisheries 7: 331-363.

O'Neill, R.J.W., M.J. O'Neill and J.A.M. Graves (1998) Undermethylation associated with retroelement activation and chromosome remodelling in an interspecific mammalian hybrid. Nature 393: 68-72.

Ono, H., E. hirose, K. Miyazaki, H. Yamamoto and J. Matsumoto (1997). Transgenic medaka fish bearing the mouse tyrosinase gene: expression and transmission of the transgene following electroporation of the orange-colored variant. Pigment Cell Res. 10: 168-175.

Ozato, K., K. Inoue and Y. Wakamatusu (1989). Transgenic fish: Biological and technical problems. Zool. Sci. 6: 445-457.

Ozato, K., H. Kondoh, H. Inohara, T. Iwamatsu, Y. Wakamatusu and T.S. Okada (1986). Production of transgenic fish: introduction and expression of chicken δ-crystallin gene in medaka embryos. Cell Differ. 19: 237-244.

Palmiter, R.D., R.D. Brinster, R.E. Hammer, M.E. Trumbauer, M.G. Rosenfeld, N.E.. Birnberg, and R.M. Evans (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. Nature 300: 611-615.

Parfit, M. (1995). Diminishing returns: Exploiting the ocean's bounty. Natl. Geographic. 185 (5): 2-37.

Pati, S., S. Mirkin, B. Feuerstein and D. Zarling (1997). Sequence-specific DNA targeting. (1997). Ency. Cancer 3: 1601-1625.

Patil, J.G. and H.W. Khoo (1995). Ultrastructural in situ hybridization and autoradiographic detection of foreign DNA in zebrafish spermatozoa.Biochem. Mol. Biol. Int. 35: 965-969.

Patil, J.G. and H.W. Khoo (1996). Nuclear internalization of foreign DNA by zebrafish spermatozoa and its enhancement by electroporation. J. Exp. Zool. 274: 121-129.

Patil, J.G., V. Wong and H.W. Khoo (1994). Assesment of pMTL construct for detection in vivo of luciferase expression and fate of the transgene in the zebrafish, *Brachydanio rerio*. Zool. Sci. 11: 63-68.

Pawlowski, W.P. and D.A. Somers (1998). Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA. Proc. Natl. Acad. Sci. USA 95: 12106-12110.

Penman, D.J., A.J. Beeching, S. Penn and N. Macklean (1990). Factors affecting survival and integration following microinjection of novel DNA into rainbow trout eggs. Aquaculture 85: 35-50.

Penman, D.J., A. Iyengar, A.J. Beeching, A. Rahman, Z. Sulaiman, and N. Maclean (1991). Patterns of transgene inheritance in rainbow trout (*Oncorhynchus mykiss*). Mol. Reprod. Dev. 30: 201-206.

Perry, A.C.F., T. Wakayama, H. Kishikawa, T. Kasai, M. Okabe, Y. Toyada, and R. Yanagimachi (1999). Mammalian transgenesis by intracytoplasmic sperm injection. Science 284: 1180-1183.

Peters, K.G., P.S. Ro, B.S. Bell, and L.A. Kindman (1995). Green fluorescent fusion proteins: powerful tools for monitoring protein expression in live zebrafish embryos. Dev. Biol. 171: 252-257.

Phi-Van, L., J.P. von Kries, W. Ostertag, and W.H. Stratling (1990). The chicken lysozyme 5' matrix attachment region increases transcription from a heterologous promoter in heterologous cells and dampens position effects on the expression of transfected genes. Mol. Cell. Biol. 10: 2302-2307.

Pikaart, M.J., F. Recillas-Targa and G. Felsenfeld (1998) Loss of transcriptional activity of a transgene is accompanied by DNA methylation and histone deacetylation and is prevented by insulators. Genes Dev. 12: 2852-2862.

Pinkert, C.A. and J.D. Murray (1999). Transgenic farm animals. *IN* Murray, J.D., G.B. Anderson, A.M. Oberbauer and M.M. McGlouoghlin (eds.) *Transgenic Animals in Agriculture*. CABI Publishing, Wallingford, UK. pp. 1-18.

Plasterk, R.H. (1993). Molecular mechanisms of transposition and its control. Cell 74: 781-786.

Pollock, R. and M. Gilman (1997). Transcriptional activation: is it rocket science? Proc. Natl. Acad. Sci. USA 94: 13388-13389.

Poon, R., F. Siong, M. Shears, G. Fletcher, and C.L. Hew (1999). Liver-specific and seasonal expression of flounder anti-freeze protein transgene in Atlantic salmon. Transgenic Res. (submittted).

Postlethwait, J., S. Johnson, C.N. Midson, W.S. Talbot, M. Gates, E.W. Ballenger, D. Africa, R. Andrews, T. carl, J.S. Eisen, S. Horne, C.B. Kimmel, M. Hutchinson, M. Johnson, and A. Rodriguez (1994). A genetic map for the zebrafish. Science 264: 699-703.

Postlethwait, J., A. Amores, A. Force, and Y-L. Yan (1999). The zebrafish genome. *IN* Detrich, H.W., M. Westerfield, and L.I. Zon (eds.) *The Zebrafish: Genetics and Genomics* Meth. Cell Biol. 60: 149-163.

Powers, D.A., T.T. Chen and R. Dunham (1992a). Transgenic Fish *IN* Murray, J.A.H. (ed.) *Transgenesis. Applications of Gene Transfer.* John Wiley and Sons, NY. pp. 233-249.

Powers, D.A., L. Hereford, T. Cole, T.T. Chen, C.M. Lin, K. Kight, K. Creech, and R. Dunham (1992b). Electroporation: a method for transferring genes into genes of zebrafish (*Brachydanio rerio*), channel catfish (*Ictalurus punctatus*) and common carp (*Cyprinus carpio*). Mol. Mar. Biol. Biotech. 1: 301-308.

Price-Haughey, J., K. Bonham and L. Gedamu (1987). Metallothionein gene expression in fish cell lines: its activation in embryonic cells by 5-azacytidine. Biochim. Biophys. Acta 908: 158-1568.

Prioleau, M.N., J. Huet, A. Sentenac, and M. Méchali (1994). Competition between chromatin and transcription complex assembly regulates gene expression during early development. Cell 77: 439-449.

Radice, A.D., B. Bugaj, D. Fitch, and S.W. Emmons (1994). Widespread occurrence of the Tc1 transposon family: Tc1-like transposons from teleost fish. Mol. Gen. Genet. 244: 606-612.

Rahman, M.A. and N. Maclean (1992a). Production of transgenic tilapia (*Oreochromis niloticus*) by one-cell stage microinjection. Aquaculture 105: 219-233.

Rahman, A. and N. Maclean (1992b) Fish transgene expression by direct injection into fish muscle. Mol. Mar. Biol. Biotech. 1: 286-289.

Rahman, A. and N. Maclean (1998a). Expression of a novel piscine growth hormone gene results in growth enhancement in transgenic tilapia (*Oreochromis niloticus*). Transgenic Res. 7: 357-369.

Rahman, A. and N. Maclean (1998b). Production of lines of growth enhanced transgenic tilapia (*Oreochromis niloticus*) expression a novel piscine growth hormone gene. *IN* LeGal, Y. and H.O. Halvorson (eds.) *New Developments in Marine Biotechnology*, Plenum Press, N.Y. pp. 19-28.

Rand-Weaver, M. and H. Kawauchi (1993). Growth hormone, prolactin and somatolactin: a structural overview. *IN Biochemistry and Molecular Biology of Fishes* Hochachka, P.W. and T.M. Mommsen (eds.) Elsevier, Amsterdam. Vol. 2, pp. 39-56.

Rauber, A. (1886). Personaltheil und germinaltheil des individuum. Zool. Anz. 9: 166-171.

Raz, E., D.A. Carson, S.E. Parker, T.B. Parr, A.M. Abai, G. Aichinger, S,W. Gromowski, M. Singh, D. Lew, M.A. Yankauckas, S.M. Baird and H.H. Rhodes (1994). Intradermal gene immunization: The possible role of DNA uptakein the induction of cellular immunity to viruses. Proc. Natl. Acad. Sci. USA 91: 9519-9523.

Raz, E., H.G.A.M. van Luenen, B. Schaerringer, R.H.A. Plasterk and W. Driever (1998). Transposition of the nematode *Caenorhabditis elegans Tc3* element in the zebrafish *Danio rerio*. Curr. Biol. 8: 82-88.

Reinhard, E., E. Nedivi, J. Wegner, J.H.P. Skene, and M. Westerfield (1994). Neural selective activation and temporal regulation of a mammalian GAP-43 promoter in zebrafish. Development 120: 1767-1775.

Riggs, A.D. (1975). X inactivation, differentiation, and DNA methylation. Cytogenet. Cell Genet. 14: 9-25.

Rinder, H., T.A. Bayer, E.M. Gertzen and W. Hoffmann (1992). Molecular analysis of the ependymin gene and functional test of its promoter region by transient expression in *Brachydanio rerio*. DNA Cell Biol. 11: 425-432.

Robinson H.L., H.S. Ginsberg, H.L. Davis, S.A. Johnston and M.A. Liti (1997). The scientific future of DNA for immunization. http://www.asmusa.org/acasrc/aca1/htm.

Rokkones, E., P. Aleström, H. Skjervold and K.M. Gautvik (1985). Development of a technique for microinjection of DNA into salmonid eggs. Acta Physiol. Scand 124, [Suppl 542]: 417.

Rokkones, E., P. Aleström, H. Skjervold and K.M. Gautvik (1989). Microinjection and expression of a mouse metallothionein human growth hormone fusion gene in fertilized salmonid eggs. J. Comp. *Physiol. B.* 158: 751-758.

Roth, L.W.A., P. Bormann, A. Bonnet and E. Reinhard (1999). β-Thymosin is required for axonal tract formation in developing zebrafish brain. Develop. 126: 1365-1374.

Roughgarden, J. and F. Smith (1996). Why fisheries collapse and what to do about it. Proc. Natl. Acad. Sci. USA 93: 5078-5083.

Sato, A., J. Komura, P. Masahito, S. Matsukuma, K. Aoki, and T. Ishikawa (1992). Firefly luciferase gene transmission and expression in transgenic medaka (*Oryzias latipes*). Mol. Mar. Biol. Biotech. 1: 318-325.

Schartl, M., Gómez, A., and Hong, Y. (1998). Gene transfer in fish: approaches, progress and perspectives, Third European Marine Science and Technology Conference Proceedings, Lisbon.

Schmidt, K.F. (1997). 'No-take' zones spark fisheries debate. Science 277: 489-491.

Schulte, P.M., D.A. Powers and M. Schartl (1998). Efficient gene transfer into Xiphorphorus muscle and melanoma by injection of supercoiled plasmid DNA. Mol. Mar. Biol. Biotech. 7: 241-247.

Schwab, M., J. Vielkind and F. Anders (1976). An approach to genetic transformation in the Xiphophorine fish. Mol. Gen. Genet. 144: 151-158.

ScienceScope (1998). Fencing over swordfish. Science 282: 1619.

Sekine, S., T. Mizukami, T. Nishi, Y. Kuwana, A. Saito, M. Sato, S. Itoh, and H. Kawauchi (1985). Cloning and expression of cDNA for salmon growth hormone in *E. coli*. Proc. Natl. Acad. Sci. USA 82: 4306-4310.

Sekkali, B., A. Belayew, J.A. Martial, B.A. Hellemans, F. Ollevier, and F.A. Volckaert (1994). A comparative study of reporter gene activities in fish cells and embryos. Mol. Mar. Biol. Biotech. 3: 30-34.

Sena, E.P. and D. A. Zarling (1993). Targeting in linear DNA duplexes with two complementary probe strands for hybrid stability. Nature Genetics 3: 365-371.

Sharps, A., K. Nishiyama, P. Collodi and David Barnes (1992). Comparison of activities of mammalian viral promoters directing gene expression in vitro in zebrafish and other fish cell lines. Mol. Mar. Biol. Biotech. 1: 426-431.

Shears, M.A., G.L. Fletcher, C.L. Hew, S. Gauthier and P.L. Davies (1991). Transfer, expression, and stable inheritance of antifreeze protein genes in Atlantic salmon (*Salmo salar*). Mol. Mar. Biol. Biotech. 1: 58-63.

Shigekawa, K. and W.J. Dower (1988). Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. Biotechniques 6: 742-751.

Simmen, M.W., S. Leitgeb, J. Charlton, S.J.M. Jones, B.R. Harris, V.H. Clark, and A. Bird (1999). Nonmethylated transposable elements and methylated genes in a chordate genome. Science 283: 1164-1167.

Simpson, E.M.; C.C. Linder, E.E Sargent, M.T. Davisson, L.E. Morbraaten and J.J. Sharp (1997). Genetic variation among 129 substrains and its importance for targeted mutagenesis in mice. Nature Genetics 16: 19- 27.

Sin, F.Y.T., A.L. Bartley, S.P. Walker, I.L. Sin, J.E. Symonds, L. Hawke and C.L. Hopkins (1993). Gene transfer in chinook salmon (*Oncorhynchus tshawytscha*) by electroporating sperm in the presence of pRSVlacZ DNA. Aquaculture 117: 57-69.

Sin, F.Y.T., J.G.I. Khoo, U.K. Mukherjee, and I.L. Sin (1998). Retention of a foreign gene transferred as a protamine-DNA complex by electroporated salmon sperm. *IN* LeGal, Y. and H.O. Halvorson (eds.) *New Developments in Marine Biotechnology*, Plenum Press, N.Y. pp. 29-31.

Skyrud, T., O. Anderson, P. Aleström, and K.M. Gautvik (1989). Effects of recombinant human growth hormone and insulin-like growth factor 1 on body growth and blood metabolites in brook trout (*Salvelinus fortinalis*). Gen. Comp. Endocrinol. 75: 247-255.

Smith, K.T., A.J. Shepard, J.E. Boyd and G.M. Lees (1996). Gene delivery systems for use in gene therapy: an overview of quality assurance and safety issues. Gene Therapy 3: 190-200.

Speksnijder, J.E., W.J. Hage, P.H. Lanser, P. Collodi and D. Zivkovic, (1997). In vivo assay for the developmental competence of embryo-derived zebrafish cell lines. Mol Mar Biol Biotech. 6: 21-32.

Spurgeon, D. (1997). Canada's cod leaves science in hot water. Nature 386: 105-107.

Stainier, D.Y.R., B. Fouquet, J-N. Chen, K.S. Warren, B.M. Weinstein, S.E. Meiler, M-A.P.K. Mohideen, S.C.F. Neuhauss, L. Solnica-Krezel, A.F. Schier, F. Zwartkruis, D.L. Stemple, J. Malicki, W. Driever, and M.C. Fishman (1996). Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. Development 123: 285-292.

Stavnezer, E., Barkas, A.E., Brennan, L.A., Brodeur, D., and Li, Y. (1986). Transforming Sloan-Kettering viruses generated from the cloned v-*ski* oncogene by *in vitro* and *in vivo* recombinations. J. Virol. 57: 1073-1083.

Streisinger, G., C. Walker, N. Dower, D. Knauber and F. Singer (1981). Production of clones of homozygous diploid zebra fish (*Brachydanio rerio*). Nature 291: 293-296.

Struhl, K. (1998). Histone acetylation and transcriptional regulatory mechanisms. Genes Dev. 12: 599-606.

Stuart, G.W., J.V. McMurray and M. Westerfield (1988). Replication, integration, and stable germ-line transmission of foreign sequences injected into early zebrafish embryos. Development 103: 403-412.

Stuart, G.W., J.R. Vielkind, J.V. McMurray and M. Westerfield (1990). Stable lines of transgenic zebrafish exhibit reproduction patterns of transgene expression. Development 109: 577-584.

Sun, L., S. Bradford, S. and D.W. Barnes (1995a). Feeder cell cultures for zebrafish embryonal cells in vitro. Mol Mar Biol Biotech. 4: 43-50.

Sun, L., C.S. Bradford, C. Ghosh, P. Collodi, and D.W. Barnes (1995b). ES-like cell cultures derived from early zebrafish embryos. Mol. Mar. Biol. Biotech. 4: 193-199.

Sutrave, P., Kelly, A.M., and Hughes, S.H. (1990). *ski* can cause selective growth of skeletal muscle in transgenic mice. Genes Dev. 4: 1462-1472.

Symonds, J.E., S.P. Walker and F.Y.T. Sin (1994). Electroporation of salmon sperm with plasmid DNA: evidence for enhanced sperm/DNA association. Aquaculture 119: 313-327.

Szelei, J. and E. Duda (1989). Entrapment of high-molecular-mass DNA molecules in liposomes for the genetic transformation of animal cells. Biochem. J. 259: 249-553.

Szelei, J., L. Váradi, F. Müller, F. Erdélyi, L. Orbán, L. Horváth, and E. Duda (1994). Liposome mediated gene transfer in fish embryos. Transgenic Res. 3: 116-119.

Takagi, S., T. Sasado, G. Tamiya, K. Ozato, Y. Wakamatsu, A. Takeshita, and M. Kimura (1994). An efficient expression vector for transgenic medaka construction. Mol. Mar. Biol. Biotech. 3 192-199.

Tamiya, E., T. Sugiyama, K. Masaki, A. Hirose, T. Okoshi and I. Karube (1990). Spatial imaging of luciferase gene expression in transgenic fish. Nucl. Acids Res. 18: 1072.

Tan, J-H. and W-K. Chan (1997). Efficient gene transfer into zebrafish skeletal muscle by intramuscular injection of plasmid DNA. Mol. Mar. Biol. Biotech. 6: 98-109.

Tang D. C., M. de Vit and S.A. Johnston (1992). Genetic immunization is a simple method for eliciting an immune response. Nature; 356: 152-154.

Tazi, J. and A. Bird (1990). Alternative chromatin structure at CpG islands. Cell 60: 909-920.

Tewari, R., C. Michard-Vahee, E. Parrot, and D. Chourrout (1992). Mendelian transmission, structure and expression of transgenes following their injection into the cytoplasm of trout eggs. Transgenic Res. 1: 250-260.

Thomas, K.R., C. Deng, and M.R. Capecchi (1992). High-fidelity gene targeting in embryonic stem cells by using sequence replacement vectors. Mol. Cell. Biol. 12: 2919-2923.

Toyohara, H, T. Nakata, K. Touhata, H. Hashimoto, M. Kinoshita, M. Sakaguchi, M. Nishikimi, K. Yagi, Y. Wakamatsu and K. Ozato (1996). Transgenic expression of L-gluono-γ-lactone oxidase in medaka (*Oryzias latipes*), a teleost fish that lacks this enzyme necessary for L-ascorbic acid biosynthesis. Biochem. Biophys. Res. Comm. 223: 650-653.

Tsai, H-J., S.H. Wang, K. Inoue, S. Takagi, M. Kimura, Y. Wakamatsu, and K. Ozato (1995a). Initiation of transgenic *lacZ* gene expression in medaka (*Oryzias latipes*) embryos. Mol. Mar. Biol. Biotech. 4: 1-9.

Tsai, H-J., F.S. Tseng and I.C. Liao (1995b). Electroporation of sperm to introduce foreign DNA into genome of loach (*Misgurnus anguillicaudatus*). Canad. J. Fish. Aquat. Sci. 52: 776-787.

Tsunoda, Y., T. Tokunaga, H. Imai and T. Ochida (1989). Nuclear transplantation of male primordial germ cells in the mouse. Development 107: 407-411

Tung, T.C., Chang, C. Y., and Tung, Y. F. Y. (1945). Experiments on the development potencies of blastoderm and fragment of teleostean egg separated latitudinally. Proc Zool Soc Lond 115: 175-188.

Tung, T. C., Wu, S. C., Tung, Y. Y. F., Yen, S. S., Tu, M., and Lu, T. Y. (1965). Nuclear transplantation in fishes. Scientia Sinica (Peking) 14: 1244-1245.

Tweedie, S., K. Charlton, V. Clark, and A. Bird (1997). Methylation of genomes and genes at the invertebrate-vertebrate boundary. Mol. Cell. Biol. 17: 1469-1475.

Ueno, K., S. Hamaguchi, K. Ozato, J.H. Kang and K. Inoue (1994). Foreign gene transfer into nigorobuna (*Carassius auratus grandoculis*). Mol. Mar. Biol. Biotech. 3: 235-242.

Ulmer, J.B., J.J. Donnelly, and M.A. Liu (1996). DNA vaccines promising: A new approach to inducing protective immunity. ASM News 62: 476-479.

Vaughan, L., R. Fitzpatrick, B. McKenna, K. Phenix, D. Todd, L. Reitan, A. Illehaug, A. Ramstad and P. Liljestro (1998). Genetic Immunization of Fish. Third European Marine Science and Technology Conference Proceedings, Lisbon.

Vielkind, J., U. Vielkind, E. von Grotthuss, and F. Anders (1971). Uptake of bacterial H3-DNA into fish embryos. Experientia 27: 347-348.

Vielkind, J., H. Haas-Abdekam, U. Vielkind and F. Anders (1982). The induction of a specific pigment cell type by total genomic DNA injected into the neural crest region of fish embryos of geneus *Xiphophorus*. Mol. Gen. Genet. 185: 379-389.

Vielkind, J.R. (1992) Medaka and zebrafish: ideal as transient and stable transgenic systems. *IN* Hew, C.L. and G.L. Fletcher (eds.) *Transgenic Fish*. World Scientific Publishers, Singapore. pp. 72-91.

Volckaert, F.A., B.A. Hellemans, P. Galbusera, F. Ollevier, B. Sekkali, and A. Belayew (1994). Replication, expression and fate of foreign DNA during embryonic and larval development of the African catfish (*Clarias gariepinus*). Mol. Mar. Biol. Biotech. 3: 57-69.

Voltaire, F-M. A. (1759). *Candide*. Various publishers, e.g., Oxford University Press.

von Hipple, P.H. (1998). An integrated model of the transcription complex in elongation, termination and editing. An integrated model of the transcription complex in elongation, termination and editing. Science 281: 660-665.

von Melcher, H., S. Reddy, and H.E. Ruley (1990). Isolation of cellular promoters by using a retrovirus promoter trap. Proc. Natl. Acad. Sci. USA 87: 3733-3737.

Vos, J. C., De Baere, I., and Plasterk, R. H. (1996). Transposase is the only nematode protein required for in vitro transposition of Tc1. Genes Dev. 10: 755-761.

Wada, H., K. Nasruse, A. Shimada, and A. Shima (1995). Genetic linkage map of a fish, the Japanese medaka *Oryzias latipes*. Mol. Mar. Biol. Biotech. 4: 269-274.

Wakamatsu, Y., K. Ozato, H. Hashimoto, M. Kinoshita, M. Sakaguchi, Y. Hyodo-Taguchi, and H. Tomita (1993). Generation of germ-line chimeras in medaka (*Oryzias latipes*). Mol. Mar. Biol. Biotech. 2: 325-332.

Wakamatsu, Y., K. Ozato and T. Sasado (1994). Establishment of pluripotent cell line derived from a medaka (*Oryzias latipes*). Mol. Mar. Biol. Biotech. 3: 185-191.

Wang, R., P. Zhang, Z. Gong and C.L. Hew (1995). Expression of the antifreeze protein gene in transgenic goldfish (*Carassius auratus*) and its implications in cold adaptation. Mol. Mar. Biol. Biotech. 4: 20-26.

Warren, K.S. and M.C. Fishman (1998). "Physiological genomics": mutant screens in zebrafish. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H1-H7.

Weinberg, E.S. (1998). Zebrafish genetics: Harnessing horizontal gene transfer. Curr. Biol. 8: R244-247.

Weinstein, B.M., A.F. Schier, S. Abdelilah, J. Malicki, L. Solnica-Krezel, D.L. Stemple, D.Y.R. Stainier, F. Zwartkruis, W. Driever and M.C. Fishman (1996). Hematopoietic mutations in the zebrafish. Development 123: 303-309.

Westerfield, M., J. Wegner, B.G. Jegalian, E.M. DeRobertis, and A.W. Püschel (1992). Specific activation of mammalian *Hox* promoters in mosaic transgenic zebrafish. Genes Dev. 6: 591-598.

Whalen RG. (1998). DNA Vaccine Web. http://www.genweb.com/dnavax.

Wilmut, I., A.E. Schnieke, J. McWhir, A.J. Kind and K.H.S. Campbell (1997). Viable offspring derived from fetal and adult mammalian cells. Nature 385: 810-813.

Williams, D.W., F. Müller, F.L. Lavender, L. Orbán and N. Maclean (1996). High activity in the yolk syncytial layer affects quantitative transient expression assays in zebrafish embryos. Transgenic Res. 6: 433-442.

Winkler, C., J.R. Vielkind and M. Schartl (1991). Transient expression of foreign DNA during embryonic and larval development of the medaka fish (*Oryzias latipes*). Mol. Gen. Genet. 226: 129-140.

Winkler, C., Y. Hong, J. Wittbrodt and M. Schartl (1992). Analysis of heterologous and homologous promoters and enhancers *in vitro* and *in vivo* by gene transfer into Japanese medaka (*Oryzias latipes*) and *Xiphophorus*. Mol. Mar. Biol. Biotech. 1: 326-337.

Winkler, C., J. Wittbrodt, R. Lammers, A. Ulrich and M. Schartl (1994). Ligand-dependent tumor induction in medakafish embryos by a *Xmrk* receptor tyrosine kinase transgene. Oncogene 9: 1517-1525.

Winkler, C. and M. Schartl (1997). Gene transfer in laboratory fish: Model organisms for the analysis of gene function. *IN* Houdebine, L.M. (ed.) *Transgenic Animals: Generation and Use*. Harwood Academic Publishers, Amsterdam. pp. 387-395.

Wittbrodt, J., A. Meyer, and M. Schartl (1998). More genes in fish? BioEssays 20: 511-515.

Wolf, K. (1988). *Fish Viruses and Fish Viral Diseases*. Ithaca, NY: Cornell University Press.

Wu, T., H. Yang, Z. Dong, D. Xia, Y. Shi, X.Ji, Y. Shen and W. Sun (1994). The integration and expression of human growth hormone gene in blunt nout bream and common carp. J. Fish. China, Shuichan Xuebao 18: 284-289.

Wu, S.M., P.P. Hwang, C.L. Hew and J.L. Wu (1998). Effect of antifreeze protein on cold tolerance in juveniles of tilapia (*Oreochromis mossambicus Peters*) and milkfish (*Chanos chanos Forsskal*). J. Zoological Studies 37: 39-44.

Xie, Y., X. Chen and T.E. Wagner (1997). A ribozyme-mediated gene "knockdown" strategy for the identification of gene function in zebrafish. Proc. Natl. Acad. Sci. USA 94: 13777-13871.

Xie, Y., D. Liu, J. Zou, G. Lie, and Z. Zhu (1989). Novel gene transfer in the fertilized eggs of loach via electroporation. Acta Hydorbiologica Sinica 13: 387-389.

Xiong, F., R.A. Chin and C.L. Hew (1992). A gene encoding chinook salmon (*Oncorhynchus tschawytscha*) prolactin: gene structure and potential *cis*-acting regulatory elements. Mol. Mar. Biol. Biotech. 1: 155-164.

Yamaha, E., H. Onozato and F. Yamazaki (1988a). Visualization of female pronucleus in the goldfish, Carassius auratus, using fluorescent dye Hoechst 33342. *Nippon Suisan Gakkaishi* 54: 537.

Yamaha, E., E. Matsuoka and F. Yamazaki (1988b). Introduction of exotic reagents into denuded eggs of goldfish and crucian carp by electroporation. *Nippon Suisan Gakkaishi* 54: 2043.

Yan, S.Y. (1989). The nucleo-cytoplasmic interactions as revealed by nuclear transplantation in fish. *IN* Malacinsky, G.M. (ed.) *Cytoplasmic Organization System.* McGraw-Hill. NY. pp. 61-81.

Yan, S.Y., M. Tu, H. Yang, Z. Mao, Z. Zhao, L. Fu, G. Li, G. Huang, S. Li, G. Jin, L. Ma, L. Lin, S. Xia and L. Yu (1990). Developmental incompatibility between cell nucleus and cytoplasm as revealed by nuclear transplantation experiments in teleost of different families and orders. Int. J. Dev. Biol. 34: 255-265.

Yan, S. (1998). *Clonning in Fish - Nucleocytoplasmic Hybrids.* IUBS Educational and Cultural Press Ltd., Hong Kong.130.

Yan, S.Y., Z.R. Mao, H.Y. Yang, M. Tu, S.H. Li, G.P.I. Huang, G.S. Li, L. Guo and R.F. He (1991). Further investigation on the nuclear transplantation in different orders of teleost: the combination of the nucleus of tilapia (*Oreochromis nilotica*) and the cytoplasm of loach (*Paramisgurmus dabtyanus*). Int. J. Dev. Biol. 35: 429-435.

Yoder, J.A., C.P. Walsh, and T.H. Bestor (1997). Cytosine methylation and the ecology of intragenomic parasites. Trends Genet. 13: 335-340.

Yoon, S.J., E.M. Hallerman, M.L. Gross, Z. Liu, J.F. Schneider, A.J. Faras, P.B. Hackett, A.R. Kapuscinski and K.S. Guise (1990). Transfer of the gene for neomycin resistance into goldfish, *Carassius auratus*. Aquaculture 85: 21-33.

Yoshizaki, G., T. Oshiro, and E. Takashima (1991). Introduction of carp α-globin gene into rainbow trout. Nippon Suisan Gakkaishi 57: 819-824.

Yuh, C-H. H. Boplouri and E.H. Davidson (1998) Genomic *cis*-regulatory logic: Experimental and computational analysis of a sea urchin gene. Science 279: 1896-1902.

Zafarullah, M., P.E. Olsson and L. Gedamu (1990). Differential regulation of metallothionein genes in rainbow trout fibroblasts, RTG-2. Biochim. Biophys. Acta 1049: 318-323.

Zanta, A. M., P. Belguise-Valladier and J.-P. Behr (1999). Gene delivery: A single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. Proc. Natl. Acad. Sci. USA 96: 91-96.

Zelenin, A.V., A.A. Alimov, V.A. Barmintzev, A.O. Beniumov, I.A. Zelenina, A.M. Krasnov, V.A. Kolesnikov (1991). The delivery of foreign genes into fertilized fish eggs using high-velocity microprojectiles. FEBS Lett. 287: 118-120.

Zhang, P., M. Hayat, C. Joyce, L.I. Gonzalez-Villasenor, C.M. Lin, R.A. Dunham, T.T. Chen, and D.A. Powers. (1990). Gene transfer, expression and inheritance of pRSV-rainbow trout-GH cDNA in the common carp, *Cyprinus carpio* (Linnaeus) . Mol. Rep. Dev. 25: 3-13.

Zhang, N. et al. (1990). Nuclear transplantation between different cyprind genera and subfamilies using donor nuclei from cultured cells of grass carp and black carp. Acta Fish Sci. 14: 344-346.

Zhang, P., Y. Xu, Z. Liu, Y. Xiang, S. Du and C.L. Hew (1998). Gene transfer in red sea bream (*Pagrosomus major*). *IN* LeGal, Y. and H.O. Halvorson (eds.) *New Developments in Marine Biotechnology*, Plenum Press, N.Y. pp. 15-18.

Zhang, P., J. Zhou and T. Wang (1993). Gene transfer in goldfish, *Carassius auratus*, by oocyte microinjection. Aquaculture 111: 311.

Zhao, X., P.J. Zhang and T.K. Wong (1993). Application of Baekonization: a new approach to produce transgenic fish. Mol. Mar. Biol. Biotech. 2: 63-69.

Zhu, Z., G. Li, L. He, S. Chen (1985). Novel gene transfer into the fertilized eggs of goldfish (*Carassius auratus* L. 1758). Z. Angew. Ichthyol. 1: 31-34.

Zhu, Z., Hm Xu, G. Li, Y. Xie and L. He (1986). Biological effects of human growth hormone gene microinjected into the fertilized eggs of loach *Misgurnus anguillicaudatus* (Cantor). Kexue Tongbao 31: 988-990.

Zhu, Z. (1992). Generation of fast growing transgenic fish: methods and mechanisms. *IN* Hew, C.L. and G.L. Fletcher (eds.) *Transgenic Fish*. World Scientific Publishers, Singapore. pp. 92-119.

Zietkiewicz, E., M. Labuda, D. Sinnett, F.H. Glorieux and D. Labuda, (1992). Linkage mapping by simultaneous screening of multiple polymorphic loci using Alu oligonucleotide-directed PCR. Proc. Natl. Acad. Sci. USA 89: 8448-8451.

TABLE 1

Enhancer/Promoter Strengths in Cultured Fish Cell Lines

Enhancer/ Promoter[1]	Cell Line[2]	Relative Activity[3]	References
VIRAL			
RSV	EPC,A2,RTPit, goldfish ZEM1, ZEM2,	**100**	Friedenrich (90), Liu (90d), Sharps(92), Sato (92), Bétancourt (93), Sekkali (94)
CMV	EPC, ZEM2	2000	Sharps (92)
	EPC	300-800	Sekkali (94)
	EPC	80-100	Bétancourt (93), Bearzotti (92)
	ZEM1	100	Sharps (92)
	CHSE	100	Bétancourt (93)
TK	EPC, ZEM2	1000	Sharps (92)
	ZEM1	25	Sharps (92)
	EPC,A2	100	Friedenrich (90)
CMV/tk	EPC	100	Friedenrich (90)
	A2	400	Friedenrich (90)
SV40	goldfish	5	Sato (92)
	EPC	<10	Bearzotti (92)
	EPC	10-20	Friedenrich (90), Bétancourt (93)
	EPC, ZEM1	100	Liu (90d), Sharps (92)
	EPC, ZEM2	2000	Sharps (92)
	CHSE	300	Bétancourt (93)
Py/tk	EPC	100	Friedenrich (90)
	A2	10	Friedenrich (90)
CONSTITUTIVE			
cβ-actin	EPC	500	Liu (90d)
xβ-actin	EPC	30	Bearzotti (92)
	EPC, RTH149, RTG	0.5-500	Moav (92a)
INDUCIBLE			
hHS	EPC	15/50 (u/i)	Liu (90d)
	goldfish	15 (u)	Sato (92)
X47	EPC	5/6 (u/i)	Friedenrich (90)
	A2	40/50 (u/i)	Friedenrich (90), Winkler (92)
	RTH	2/3 (u/i)	Winkler (92)
hMT	EPC,A2,RTH	30/150(u/i)	Friedenrich (90), Hong (93)
	goldfish	25 (u)	Sato (92)
hMT/tk	EPC	10/120 (u/i)	Friedenrich (90)
	A2	10/100 (u/i)	Friedenrich (90)
fMT	EPC,A2,RTH	1/120 (u/i)	Hong (93), Cavari (93b)
	RTG-2	1/170 (u/i)	Zafarullah (90)
	RTL-4	1/10 (u/i)	Inoue (92a)
AFP	RTH,CHSE,CHH	15 (u)	Du (92b)
PrIRTPit		80 (u)	Xiong (92)

TABLE 1: Enhancer/promoters are broken into three groups, those from viruses of non-piscine origin, those from genes that are constitutively expressed, and those from genes whose expression can be induced. The references include just the first author; *et al.* has been omitted for space.

1) Abbreviations: Enhancers/Promoters were from the following viruses or genes (in some cases there are hybrid regulatory elements consisting of an enhancer from one source and the promoter from another, e.g., CMV-tk): RSV, Rous sarcoma virus long terminal repeat (LTR) which is taken as an arbitrary standard at 100 units of relative activity; CMV, cytomegalovirus ; TK(tk), herpes simplex virus thymidine kinase; SV40, simian virus 40 enhancer-early promoter; Py, polyoma virus enhancer-early promoter; MMTV, mouse mammary tumor virus LTR, X47, *Xiphophorus xiphidium*; c (x) β-actin, carp (*Xenopus*) β-actin; h (d) HS, human (*Drosophila*) heat-shock-70 ; h (m, rt) MT, human (mouse, rainbow trout) metallothionein; tMTb, trout metallothionein-B ; op (wf) AFP, ocean pout (winter flounder) antifreeze protein; Prl, prolactin promoter, xEF1α, *Xenopus* translational elongation factor 1α, c (rβ-cardiac) MyHC, carp (rabbit β-cardiac) myosin heavy chain; ztα (medβ)-actin, zebrafish α (medaka β) actin; GATA-1, blood-specific promoter; MoMLV LTR, Moloney murine leukemia virus long terminal repeat; cα-globin, about 1 kb of 5' flanking sequence to the carp α globin. These abbreviations are used in Tables I, II, and III.

2) Cell lines: A2, *X. xiphidium* embryonal epitheloid cells; EPC, carp epithelial cells; RTH, rainbow trout hepatoma; CHSE, chum salmon embryonic cells; CHH, chum salmon heart cells. RTPit, rainbow trout pituitary primary cells; ZEM 1 and ZEM2, zebrafish embryonic cell lines. Other cell lines have also been tested, but on a less routine basis and so are not included in this listing.

3) The RSV promoter is standardized at 100 units of relative activity. For inducible promoters either the uninduced/induced ratios (u/i) or just uninduced levels (u) are given. Note, promoters for metallothionein genes in fish can be activated by the nucleotide analogue 5-azacytidine as well as by toxic metals (Price-Haughey et al., 1987).

TABLE 2

Marker Gene Constructs for Transgenic Fish

Gene	Promoter	Fish Species	Expression[1] Fo	F1	References
lacZ	mMT-1	salmon	+	nd	McEvoy (88)
	CMV	zebrafish		+	nd Westerfield (92), Sekkali (94)
	CMV	zebrafish (E)	+	nd	Müller (93)
	CMV	carp (E)	+	nd	Müller (93)
	CMV, CMV-tk	medaka	+	nd	Winkler (94)
	CMV-tk	seabream	+	nd	Cavari (93a)
	CMV-tk	tilapia (B-MI)	+	nd	Gómez-Chiarri (96a)
	CMV-chβ-actin	medaka	+	nd	Tsai (95a)
	RSV-LTR	zebrafish		+	+ Culp (91), Nam (98a)
	RSV-LTR	zebrafish		+	nd Lin (94a)
	RSV-LTR	zebrafish (SE)	+	nd	Müller (92)
	RSV-LTR	carp (SE)	+	nd	Müller (92)
	RSV-LTR	medaka	+	nd	Winkler (94)
	RSV-LTR	trout	+	nd	Inoue (92)
	RSV-LTR	nigrobuma	+	+	Ueno (94)
	RSV-LTR	African catfish (E)	+	nd	Müller (92, 93)
	RSV-LTR	salmon (SE)	+	nd	Sin (93)
	RSV-LTR	loach (B)	+	nd	Zelenin (91)
	RSV-LTR	loach	+	+	Nam (98a)
	RSV-LTR	zebrafish, trout (B)	nd	nd	Zelenin (91)
	RSV-chβ-actin	medaka	+	nd	Tsai (95a)
	MoMLV-LTR	medaka	+	nd	Winkler (94), Tsai (95a)
	cβ-actin	zebrafish		+	nd Müller (97)
	cβ-actin	medaka	+	+	Takagi (94)
	cβ-actin	tilapia	+	+	Alam (95)
	hHS	zebrafish		+	- Bayer (92)
	SV40	carp (MI)	+	nd	Hansen (91)
	SV40-tk	zebrafish (MI)	+	nd	Tan (97)
	SV40,hMT,X47	medaka	+	nd	Winkler (94)
	ependymin	zebrafish		+	nd Rinder (92)
	mHox1.1	zebrafish		+	nd Westerfield (92)
	hHOX3.3	zebrafish		+	nd Westerfield (92)
	xEF-1α	zebrafish		+	+ Lin (94b)
	rGAP-43	zebrafish		+	nd Reinhard (94)
	cMyHC	zebrafish		+	nd Müller (97)
	rβ-cardiacMyHC	carp (MI)	+	nd	Hansen (91)
cat	CMV	zebrafish		+	nd Liu (90d), Caldovic (95)
	CMV	zebrafish		+	nd Ivics (93), Sekkali (94)
	CMV	trout	+	+	Tewari (92)
	CMV	trout (S)	-	nd	Chourrout (92)
	CMV	seabream	+	nd	García-Pozo (98)
	CMV	zebrafish (MI)	+	nd	Tan (97)
	RSV-LTR	zebrafish		+	nd Stuart (88), Liu (90d),
	RSV-LTR	zebrafish (S)	+	nd	Khoo (92)
	RSV-LTR	zebrafish (E)	+	nd	Buono (92)
	RSV-LTR	medaka	+	nd	Chong (89), Winkler (91,92), Tsai (95b)
	RSV-LTR	goldfish	+	nd	Hallerman (90)

	RSV-LTR	walleye	+	nd	Moav (92b)
	RSV-LTR	N. pike	+	+	Moav (92b), J. Schneider (unpub)
	MoMLV-LTR	medaka	+	nd	Tsai (95a)
	SV40	zebrafish		+	+ Stuart (88)
	SV40	zebrafish		+	nd Liu (90d), Ivics (93), Izsvak (97)
	SV40	medaka	+	nd	Winkler (91, 92)
	SV40	tilapia	+	nd	Indig (92)
	SV40	carp (MI)	+	nd	Hansen (91)
	cβ-actin	zebrafish		+	+ Liu (90d), Moav (92b,93), Caldovic (95,99)
	cβ-actin	goldfish	+	nd	Moav (93)
	cβ-actin	tilapia	+	nd	Rahman (92a), Maclean (92)
	cβ-actin	tilapia (MI)	+	nd	Rahman (92b)
	cβ-actin	carp	+	nd	Moav (92a)
	cβ-actin	walleye	+	+	Moav (92b)
	cβ-actin	N. pike	+	+	Moav (92b)
	cβ-actin	trout	+	nd	Iyengar (95)
	cβ-actin	loach	+	+	Nam (96b)
	cαβ-actin	medaka	+	nd	Tsai (95a)
	cMT-A	medaka	+	nd, +	Kinoshita (94, 96), Inoue (92a)
	mMT-1	medaka	+	nd, +	Inoue (92a)
	CH-MT-1	medaka	+	+	Takagi (94)
	dHS	zebrafish		+	nd Liu (90d)
	AFP	medaka	+	nd	Gong (91), Du (92b)
	rβ-cardiacMyHC	carp (MI)	+	nd	Hansen (91)
	cMyHC	carp (MI)	+	nd	Gauvry (96)
luc	CMV	zebrafish		+	nd Gibbs (94a), Sekkali (94), Collas (1996)
	CMV	zebrafish (E)	+	nd	Müller (93)
	CMV	zebrafish (MI)	+	nd	Tan (97)
	CMV	medaka	+	nd	Sato (92)
	CMV	medaka (E)	+	nd	Murakami (94)
	CMV	African catfish	+	nd	Volckaert (94)
	CMV	African catfish (E)	+	nd	Müller (93)
	CMV	trout (MI)	+	nd	Anderson (96b)
	CMV-tk	tilapia (MI)	+	nd	Gómez-Chiarri (96a)
	CMV-tk	Xiphophorus (MI)	+	nd	Schulte (98)
	RSV-LTR	zebrafish		+	nd Alestrom (92), Gibbs (94b)
	RSV-LTR	medaka	+	nd	Tamiya (90), Alestrom (92), Gibbs (94b)
	SV40	zebrafish		-	nd Gibbs (94a)
	CH-MT-1	medaka	+	nd	Sato (92)
	CH-MT-1	zebrafish	+	-	Patil (94)
	CH-MT-1	zebrafish (SE)	+	-	Patil (96)
gfp (unpub)	xEF-1α	zebrafish		+	+ Amsterdam (95, 96), Raz (98), Mohn
	zfα-actin	zebrafish			+ + Higashijima (97)
	cβ-actin	zebrafish		+	+ Mohn (unpub.)
	GATA-1	zebrafish			+ + Long (97)
	GATA-2	zebrafish			+ nd Meng (97)
	CMV	seabream	+	nd	García-Pozo (98)
	medβ-actin	medaka	+	nd	Hamada (98)
	CMV	zebrafish		+	nd Fahrenkrug (99)
neo	RSV-LTR	goldfish	+	nd	Yoon (90), Guise (92)
	RSV-LTR	N. pike	+	nd	Guise (92)

	MoMLV-LTR	zebrafish		+	nd	Ivics (93)
	TK	carp (E)	nd	nd	Müller (92)	
	SV40	zebrafish (B)	+	nd	Zelenin (91)	
hyg	SV40	zebrafish		nd	+	Stuart (88)

TABLE II: Abbreviations are the same as in Tables I. Reporter genes transferred: *lacZ*, -galactosidase; *cat*, chloramphenicol-acetyltransferase; *luc*, luciferase; *neo*, neomycin phosphotransferase II; *hyg*, hygromycin resistance – all from *E. coli;* and gfp, green fluorescent protein from the jellyfish *Aequorea victoria* (Chalfie et al., 1994) or derivatives with enhanced fluorescence (e.g., GM2, Cormack et al., 1996). All genetic constructs were delivered by microinjection unless otherwise noted in the species column: (E), electorporation; (S), sperm-mediated; (SE) sperm electroporated with transgenic DNA prior to fertilization, (MI), direct injection into muscle , and (B), high-velocity projectile bombardment. [1)]Expression: +, expression in terms of either mRNA synthesis, protein synthesis, or enzymatic activity detected; -, no expression detected; nd, expression assay not reported.

Table 3

Transgenic Constructs Introduced into Fish

Gene	Promoter	Fish Species	Expression Fo	Expression F1	References
hGH cDNA	mMT-1	goldfish	+	nd	Zhu (85)
	mMT-1	loach	+	nd	Zhu (86), Benyumov (89)
	mMT-1	loach (E)	+	nd	Xie (89)
	mMT-1	catfish	-	nd	Dunham (87)
	mMT-1	tilapia	-	nd	Brem (88)
	mMT-1	trout	+	nd	Aggelon (88)
	mMT-1	salmon	+	nd	Rokkones (89)
	mMT-1	carp	+	nd	Hernandez (91), Wu (94)
	mMT-1	carp	+	+	Zhu (92), Cui (93, 96)
	mMT-1	medaka (E)	+	nd	Lu (92)
	SV40	trout	-	nd	Chourrout (86)
	SV40	trout	+	nd	Guyomard (89)
	TK, chβ-actin	medaka (E)	+	+	Lu (92)
bGH cDNA	mMT-1	salmon	+	nd	McEvoy (88)
	RSV-LTR	walleye	+	nd	Moav (92b)
	RSV-LTR	N. pike	+	nd/+	Moav (92b), Gross (92)
	CMV-tk	seabream	+	nd	Cavari (93a)
rGH cDNA	mMT-1	salmon	-	nd	McEvoy (87)
	mMT-1	tilapia	-	nd	Rahman (92a)
	mMT-1	trout	-	+	Penman (90, 91), Maclean (92)
	cβ-actin	tilapia	+	nd	Maclean (92)
rtGH cDNA	RSV-LTR	carp	+	-	Zhang (90)
	RSV-LTR	carp	+	+	Chen (92, 93)
	RSV-LTR	carp, catfish (E)	nd	nd	Powers (92b)
	RSV-LTR	zebrafish (E)	nd	nd	Powers (92b)
	RSV-LTR	catfish	+	nd	Dunham (92)
	RmMT	medaka (E)	+	nd	Inoue (92)
csGH cDNA	opAFP	salmon	+	+	Du (92a), Devlin (94a,b, 95a,b) Rahman(98a,b), Poon(99)
	opAFP	seabream	+	nd	Zhang (98)
	opAFP	loach (SE)	nd	nd	Tsai (95b)
	cβ-actin	walleye	+	nd	Moav (92b)
	cβ-actin	N. pike	+	nd/+	Moav (92b), Gross (92)
	RSV-LTR	zebrafish (E)	+	nd	Zhao (93)
	RSV-LTR	catfish	+	nd	Dunham (92)
tiGH cDNA	RSV	tilapia	+	+	dela Fuente (1995, 1998), Hernández (97)
	CMV	tilapia	+	+	Martinez (96), Hernández (97)
sbGH cDNA	cβ-actin	trout	nd	nd	Cavari (93b)

wfAFP	wfAFP	salmon	nd	nd	Fletcher (88)
	wfAFP	salmon	+	nd	Shears (91), Hew (92)
	wfAFP	salmon	+	+	Fletcher (92), Hew (97)
	wfAFP	goldfish	+	+	Wang (95)
	RSV	tilapia	+	+	Hernández (97)
mg-Tyrs-J	mg-Tyrs-J	medaka	+	+	Matsumoto (92)
	mg-Tyrs-J	medaka (E)	+	+	Ono (97)
mTyrs	mTyrs	medaka	+	+	Hyodo-Taguchi (97)
chδ-crystalin	chδ-crystalin	medaka	+	nd	Ozato (86), Inoue (89)
Xmark	CMV-tk	medaka	+	nd	Winkler (94)
IHNV-Gprotein	CMV-tk trout	+	nd	Anderson (96)	
coIGF	csMT-1	salmon (S/E)	nd	nd	Sin (98)
cα-globin	cα-globin	trout	+	nd	Yoshizaki (91a,b)
GLO	SV40	medaka	+	+	Toyohara (96)

TABLE 3: Abbreviations are the same as in Tables 1 and 2. The following genes have been transfered into fish embryos: h (b,r,rt,cs,tr,sb) GH, human (bovine, rat, rainbow trout, chinook salmon, tilapia, seabream) growth hormone; wfAFP, winter flounder antifreeze protein; mTyrs, mouse tyrosinase; chδ-crystalin, chicken δ crystalin; IHNV-G-protein, infectious hematopoietic necrosis virus G-protein; coIGF, coho salmon insulin-like growth factor; cα-globin, carp α-globin; GLO, L-gulono-γ-lactone oxidase.

SUPPLEMENTAL POLICIES

CLASSIFICATION OF AQUACULTURE SPECIES/POPULATION AS FOOD OR NONFOOD

I. Purpose of classification: Primarily for guidance in determining enforcement priority.

II. General principles:

A. A particular aquaculture species/population is presumed to be a food species/population if it is reasonably likely that a) any significant part of the species/population will be consumed directly or indirectly by humans for food, or b) the species/population is consumed by an identifiable human population. Under this definition, incidental or inadvertent diversion of insignificant numbers to food use would not cause a species/population to be classified as food. ("Indirect" consumption refers, for example, to a species that is used as food for another aquaculture species that is in turn consumed by humans).

B. Drugs intended for use in a nonfood species/population (as defined above) are not considered to be drugs for use in a food species/population.

C. A nonfood presumption could be overcome by facts in a specific case. An example would be packaging of an alleged aquarium fish drug in commercial pond-size use packages. The possibility of diversion to food use should be considered even if the labeling warns against use in food species.

D. In general, the traditional or known use of the species involved will be the major factor in classification decisions. It should be considered, however, that some species could be used either for food or nonfood purposes. Official or authoritative species classification lists will be used whenever possible.

E. A food species/population will as a general rule be considered food at all life stages. However, the life stage will be a factor in determining enforcement priority with respect to use of a drug in a particular life stage.

III. Specific Species/Population Groups

A. Baitfish

1. Three species of baitfish (golden shiners, fathead minnows and goldfish) are considered nonfood fish. (It is reported that these species comprise 90% of all baitfish.)

2. Other species may be added to this list as support for addition is provided.

3. However, the facts related to individual population may need to be examined because, for example, some baitfish are fed to broodstock (see broodfish, below).

B. Ornamental and aquarium species

1. In general, ornamental and aquarium species are nonfood species. "Ornamental and aquarium" fish are defined as: fish that are produced and maintained solely for exhibit purposes in home or public aquaria, or in ornamental garden ponds.

2. Certain species may be cross-over species. Facts related to individual populations may need to be examined.

C. Endangered and threatened species

1. By statutory prohibition, endangered and threatened populations may not be harvested. Currently, the majority of endangered and threatened populations consist of species that are ordinarily considered to be nonfood species.

2. Approximately 110 fish species are currently listed, and limited numbers of animals are involved.

3. The Center for Veterinary Medicine has issued a letter to the U.S. Fish and Wildlife Service in which the Center specifies the circumstances under which it will exercise regulatory discretion in the use of drugs in threatened and endangered species managed by USFWS and its contractors. The principles in the letter will be applied to other agencies, Federal and State, that are responsible for managing threatened and endangered species.

D. Broodfish

1. Significant populations of some species of broodfish are consumed directly by humans. Thus, broodfish from species that are traditionally or known to be used for food are in general considered to be food fish.

2. Exceptions will be made based on facts that establish the absence of human consumption in specific species/populations of broodfish.

RELATED PROCEEDINGS APPENDIX

There are no related proceedings.